Vol. XLI, No. 1 (February, 1960), was issued on 12.2.60.

THE BRITISH JOURNAL OF EXPERIMENTAL PATHOLOGY

VOL. XLI APRIL, 1960 NO. 2

CELLULAR AND INTERCELLULAR CHANGES AFTER MECHANICAL, CHEMICAL OR RADIATION INJURY OF CONNECTIVE TISSUE*

H. Z. MOVAT†, R. H. MORE AND D. WOLOCHOW

From the Department of Pathology, Queen's University, Kingston, Canada

Received for publication July 28, 1959

THE object of the experiments to be reported was to investigate whether a plasma cellular reaction develops in the lesion which follows mechanical, chemical or physical injury of connective tissue; to find whether the fibrinoid which has been reported to develop after mechanical (incision) injury represents degenerated collagen, altered ground substance or exuded plasma proteins; to see whether fibrinoid develops following other (chemical and physical) types of non-specific injury and to determine the specificity of fibrinoid in connective tissue injury.

Plasma cells, as shown in previous publications (More and Movat, 1958, 1959*a* and 1959*b*; Movat, 1956 and Movat and More, 1955) represent the morphological equivalent of antibody formation and are therefore to be expected only after antigenic stimulation of the connective tissue. Fibrinoid occurs under a number of heterogeneous conditions. It has been reported to develop after mechanical injury and is referred to in this instance as "fibrinoid degeneration of collagen". Since we have demonstrated that the fibrinoid present in allergic injury (More and Movat, 1959*a* and 1959*b*) is the result of inspissation of exuded plasma proteins, particularly fibrin, it seemed pertinent to examine the fibrinoid material developing after mechanical injury and trace its source to either connective tissue elements or plasma constituents. In the course of these studies we also examined the other manifestations of connective tissue injury, reactivity and repair which follow non-specific injury.

MATERIALS AND METHODS

Eight New Zealand albino rabbits (Exp. I). weighing about 1800 g., were used for the study of mechanical injury. On the first day of the experiment, the right flank of each animal was shaved and painted with antiseptic. Intravenous sodium nembutal anaesthesia was

* Supported by grants-in-aid from: The National Research Council of Canada; The Canadian Arthritis and Rheumatism Society; The National Health Research Grants of Canada.

† Present address: Department of Pathology, University of Toronto, The Banting Institute, Toronto, Ontario, Canada.

given. Using sterile technique, an incision measuring approximately 2 cm. was made in the skin, deep enough to involve the subcutaneous muscle layer. The edges of the incision were opposed but not sutured and a sterile pad was placed on the incised area and firmly attached to the rabbit with adhesive tape to prevent contamination or removal by the animal.

EXPLANATION OF PLATES

FIG. 1.—Dermal lesion 24 hr. after incision. A crust (A) had formed, composed of necrotic tissue and disintegrating red blood cells. At (B) sequestration of crust by polymorphonuclear leucocytes. Haemalum-Phloxine-Saffron, × 68.

FIG. 2.—After 72 hr.: Cut edges of incision are seen lined by a black membrane of fibrinous exudate. Phosphotungstic Acid Haematin, × 25.

FIG. 3.—High-power view of fibrinous exudate seen in Fig. 2. A meshwork of fibrin is seen at (A); clumping of fibrin to form dense masses and bands of fibrinoid is seen at (B). Phosphotungstic Acid Haematin, × 175.

FIG. 4.—Another area of the fibrinous membrane seen in Fig. 2 at high power. The cut edge is infiltrated by a network of delicate threads of fibrin (A) which had precipitated along collagen fibres (B) to form bands of fibrinoid (C). The fibrin and fibrinoid seen in black in the photomicrograph was dark purplish-blue in the slide and the dark grey collagen was reddish-brown, × 175.

FIG. 5.—After 72 hr.: A thick band of intensely acidophilic fibrinoid is seen lining the cut edge of the incision. The fibrinoid seen in dark grey in the photomicrograph was intensely red in the section. Haemalum-Phloxine-Saffron, ×206.

FIG. 6.—After 5 days: Clumps and bands of fibrinoid, representing remnants of as yet unorganized fibrinous exudate, are seen in dark grey (in slide intensely orange-yellow) in the mucopolysaccharide-rich organizing connective tissue (light grey in photograph and blue in slide). Pentachrome II, × 206.

FIG. 7.—After 8 days: Acid mucopolysaccharide-rich connective tissue, containing fibroblasts, delicate reticular and collagenic fibres and occasional capillaries, fills the space of the incision. Two lymphatic-like spaces have persisted. Pentachrome II, × 206.

FIG. 8.—Lesion after 8 days still containing clumps and bands of fibrinoid and red cells. The surrounding young connective tissue contains a number of large proliferating fibroblasts. Phosphotungstic Acid Haematin, ×206.

FIG. 9.—Trypsin 2 hr.: Area of necrobiosis and lysis. There is disintegration of collagen fibres, lysis of striated muscle and infiltration of polymorphs. Haemalum-Phloxine-Saffron, ×68.

FIG. 10.—Trypsin 6 hr.: Area of lysis in sub-epidermis. Collagen fibres are disintegrating (A). The grey lakes (B) in the centre (blue in slide) represent lysed mucopolysaccharide containing reticulum fibres which normally surround fat cells. Pentachrome II, ×68.

FIG. 11.—Dermal lesion 72 hr. after injection of trypsin. An area of complete tissue necrosis is seen on top (A); in the centre there is an abscess-like zone of disintegrating polymorphonuclear leucocytes (B) and at the bottom of the photomicrograph a less damaged area or zone (C). Pentachrome II, ×25.

FIG. 12.—Marked vascular damage with exudation of a fibrinous fluid (A) around a vein and formation of fibrinoid in the vessel wall (B), as well as perivascularly (C). It is clearly visible, particularly in perivascular position, that the fibrinoid had developed from conglomeration of fibrinous exudate. Phosphotungstic Acid Haematin, ×175.

FIG. 13.—Trypsin 5 days: Fate of the fibrinous exudate seen in Fig. 12. There are several clumps and bands of fibrinoid seen at A (black in photograph and red in slide). The wavy orientation of the fibrinoid is the result of connective tissue organization. This young organizing connective tissue is composed of numerous proliferating cells, mainly fibroblasts, ground substance and young delicate fibres. Trichrome. ×206.

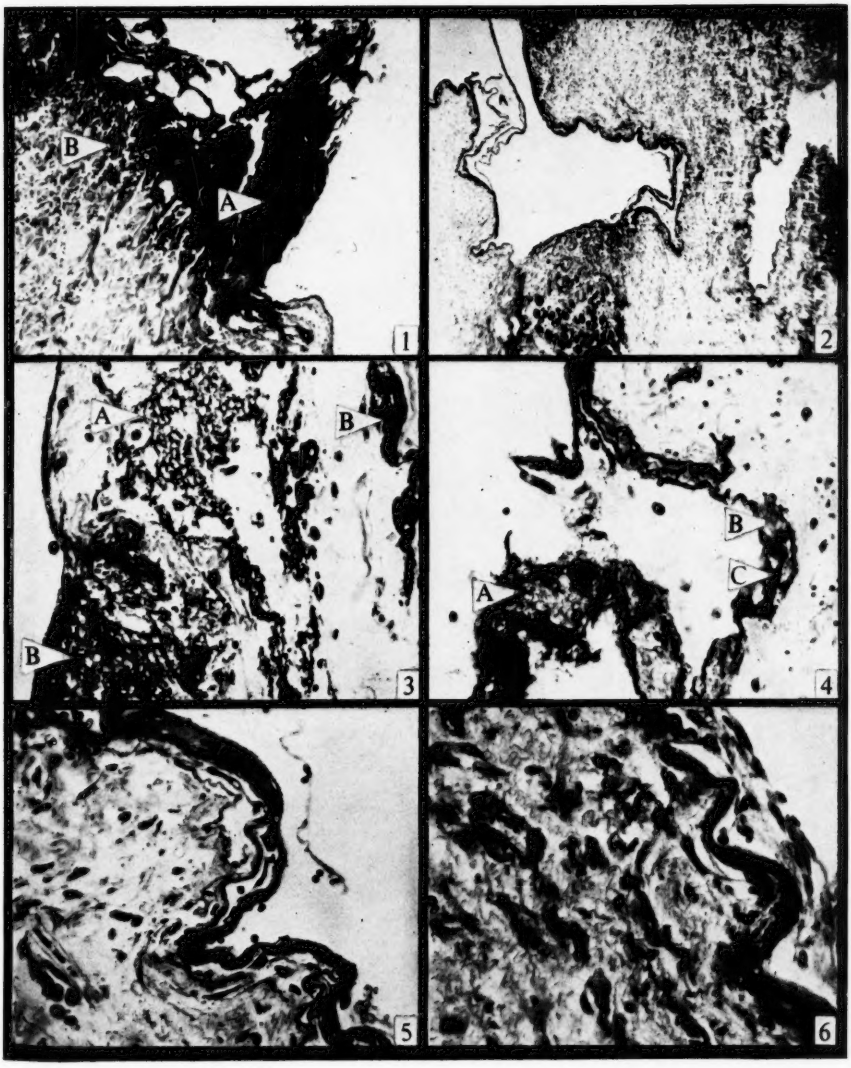
FIG. 14.—Trypsin 14 days: Formation of a homogeneous ground substance (grey in photograph, pale purplish-pink in section) between proliferating fibroblasts and newly formed capillaries. Toluidine Blue, ×170.

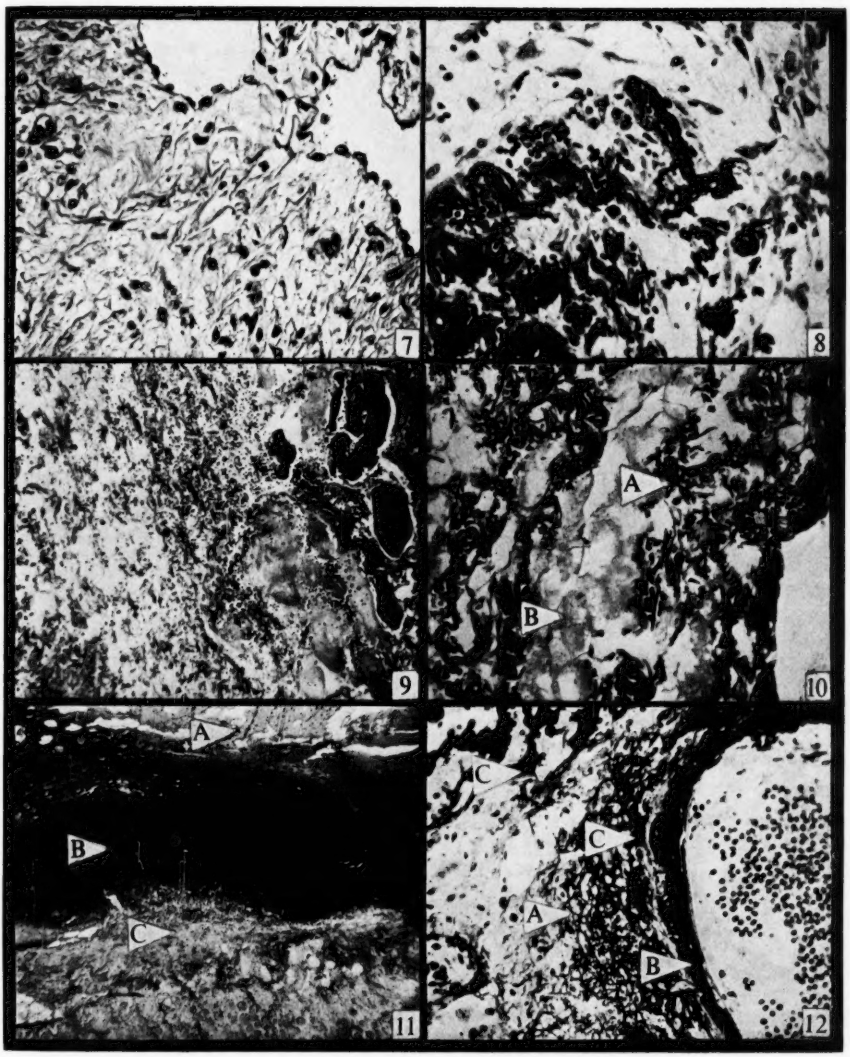
FIG. 15.—Radiation injury 18 days: Necrosis of vascular wall (A) with exudation of plasma and extension of exudate (grey in photograph and yellow in slide) in all directions (B). The collagen seen in black (C) was red in the slide. Pentachrome II, ×63.

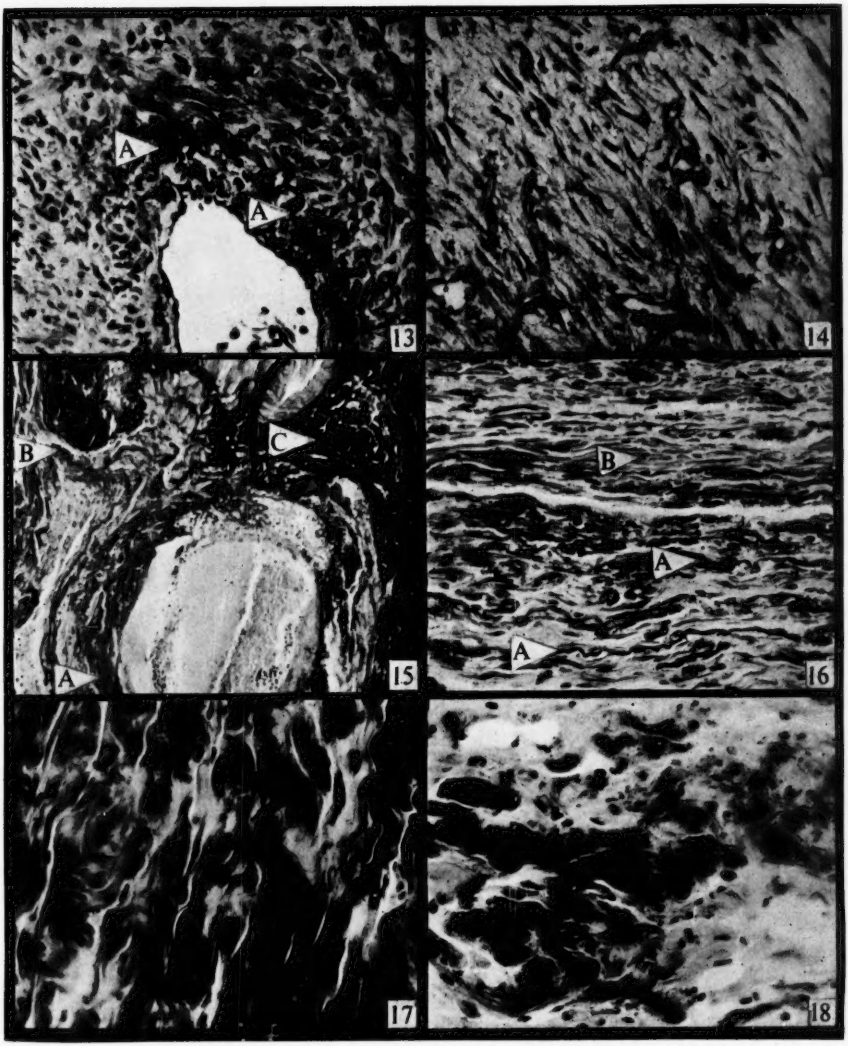
FIG. 16.—Formation of strands and bands of fibrinoid (A) (black in photograph and red in slide) between the collagen fibres (grey in photograph and blue in slide). This lesion was continuous with the exudate seen in Fig. 11. Trichrome, ×154.

FIG. 17.—Swollen dermal collagen. Pentachrome II, ×206.

FIG. 18.—Disintegrating dermal collagen. Pentachrome II, ×206.


B
A
1
2
A
B
B
3
B
C
A
4
5
6


7
8
9
A
B
10
A
B
C
11
C
C
A
B
12


A
A
13
14
B
C
A
15
B
A
A
16
17
18

The animals were killed 6, 12, 24 and 72 hr. and 5, 8 and 14 days after incision. Sections were taken at right angles to the first incision and fixed in 10 per cent buffered formaldehyde solution.

Eight rabbits (Exp. II) were injected intracutaneously on the first day with 1 ml. of a saturated solution of trypsin in distilled water. (Trypsin 1–300 obtained from National Biochemical Corp., Cleveland, Ohio.) The resulting cutaneous lesions were excised from different rabbits after the following intervals : 2, 6, 12 and 24 hr. and 3, 5, 8 and 14 days.

Three rabbits (Exp. III) were used in the final experiment. A radon seed (1·03 M.C.) was inserted subcutaneously into the right flank of each rabbit. The animals were killed 14, 18 and 30 days later. The skin where the radon seed had been inserted, which had been marked with a non-irritant dye (monastral fast blue), was excised and several strips submitted for fixation.

These tissues were processed as described previously (More and Movat, 1959 ; Movat and More, 1957).

RESULTS

Experiment I. Mechanical injury

Six hours.—A crust had formed which lay partly on the epidermis adjacent to the incision and partly in the space between the cut edges. It was composed of red blood cells, which were partly disintegrated, serum epidermis, including keratin and collagen fibres. The last stained faintly with both the cytoplasmic stains and the collagen stains. They had the appearance of the collagen seen in tissues which are inadequately fixed. There was no " fibrinoid degeneration " of collagen. A few polymorphs infiltrated the injured area. Some haemorrhage had occurred also into the tissue adjacent to the cut edge.

Twelve hours.—The space between the cut edges was almost entirely filled with blood. Some of the erythrocytes were disintegrating. Immediately adjacent to the dermal collagen, numerous strands of fibrin were seen. Oedema and haemorrhage separated the collagen fibres in some parts. Infiltration of polymorphonuclears had increased.

Twenty-four hours.—The cut edge of the skin was covered with numerous clumped erythrocytes in a meshwork of fibrin. Polymorphonuclear leucocytes, histiocytes and pseudoeosinophils were seen in the loose connective tissue in the vicinity of the incision. The crust described in the 6 hr. section was now partly demarcated by a wall of polymorphonuclear leucocytes from the adjacent healthy epidermis and dermis. This crust did not represent " fibrinoid degeneration " of collagen (Fig. 1).

Three days.—The space between the cut edges was partly filled with red cells and exudate. A sero-fibrinous exudate lined the cut edges almost throughout, presenting the appearance of a membrane (Fig. 2). This exudate stained red with Haemalum, Phloxine, Saffron (HPS) and partly dark blue with Phosphotungstic Acid Haematin (PTAH). Both the blue and red-staining varied somewhat in intensity. Delicate threads of fibrin (Fig. 3 and 4) and homogeneous bands of clumped fibrin, resembling fibrinoid (Fig. 5), stained more intensely than the granular serous exudate. Fibrin was found lining the cut edges but infiltrating also the collagen bundles adjacent to the cut edge (Fig. 4). In the PTAH the dark blue fibrin could be seen around and in between dark brown collagen fibres, but no fibrinoid degeneration of collagen fibres was demonstrable. Deeper in the dermis and subcutis, the tissue adjacent to the cut edge contained numerous proliferating fibroblasts in an acid mucopolysaccharide-rich (AMP) matrix. This matrix, which was partly homogeneous and partly fibrillar, lay between

the collagen of the dermis and a membrane of fibrin. The fibroblasts appeared active, with large nuclei, prominent nucleoli and elongated cytoplasm. They were intermingled with occasional histiocytes. A few mitotic figures were also encountered. The crust lying on the epidermis was still present.

Five days.—Sections of this specimen differed from the 3-day sections in that there was more exudate in the space between the cut edges and more newly formed AMP-rich, loose connective tissue adjacent to the cut edges. In the incision a thick layer composed of a mixture of fibrinous exudate, disintegrating red cells, AMP and proliferating cells abutted on the dermal collagen. The AMP containing young connective tissue was richer in cells than the fibrinous exudate. Most of the cells were proliferating fibroblasts with occasional histiocytes and monocytes. Where there was any extensive mass of fibrinous exudate lacking AMP material, no fibroblasts were found in the depth of this exudate. Clumps and wavy bands of fibrinoid were frequently found in the young proliferating connective tissue (Fig. 6). Fibrinoid was present also in one of the arteries adjacent to the cut edge.

A section taken from the end of the incision showed an intact but thickened epidermis with a loose cellular connective tissue underlying it. The only cells present in this lesion were fibroblasts, histiocytes and occasional polymorphs.

Eight days.—The site of incision, which corresponds to a space at 5 days, was now filled with a loose areolar connective tissue (Fig. 7). Most of the cells present in this young connective tissue were fibroblasts. These cells had abundant cytoplasm with ill-defined edges and large pale vesicular nuclei with 2–4 nucleoli. The cytoplasm was quite basophilic and vacuoles were present in a large number of cells. One had the impression that in some parts there are extended processes from one to another. Very delicate dark blue fibrils were seen in the PTAH stained slide, sometimes extending along the outside of the cytoplasm but attached to it, and occasionally extending out from it into the surrounding matrix. These seemed to be tonofibrils. The young connective tissue was composed partly of ground substance and partly of fibres. The ground substance and some of the fibres stained with the AMP stain, but quite a number of delicate fibres as well as more coarse fibres stained with the collagen stain. Occasionally newly-formed capillaries were also present in this young connective tissue. In one of the sections a circumscribed area composed of strands and clumps of fibrinoid, red cells, tissue and cellular debris and numerous macrophages was found (Fig. 8). Where the incision had severed the striated muscle there was abundant AMP-containing connective tissue with remnants of striated muscle fibres and large myogenic giant cells. These giant cells seemed to develop from the muscle nucleus and sarcoplasm after degeneration of the myofibrils. Histiocytes also proliferated in the endomysium. The cross-sectioned myofibres undergoing degeneration stained partly with the cytoplasmic stain, which ordinarily stains these structures, and partly with the AMP stain. The AMP appeared to derive from a lysis of the endomysium and possibly from the sarcolemma which also seems to stain with the AMP stains.

Fourteen days.—Grossly at this stage, the scar at the site of incision was quite small. The connective tissue was still rich in AMP, but collagen fibres were much more numerous. The fibroblasts had decreased in number and size and appeared more elongated with less abundant cytoplasm. There were also occasional macrophages, but no other cells were present in the lesion.

Experiment II. Chemical injury (injection of trypsin)

Two, six, twelve and seventeen hours.—There was a central area of necrosis which developed within 6 hr. Around this the collagen and fat disintegrated. The collagen fibres broke up and showed swelling of the interfibrillar ground substance. Fat cells in the subcutis underwent lysis and as they collapsed, the Alcian Blue (AB)-positive membrane about them formed pools of AB-positive material. The end result was a central amorphous necrotic mass. Less damage was present beyond this and the details of change that could be readily followed in this zone of less intense damage will now be described.

In this last zone two parallel processes were encountered at this stage. There was still some necrobiosis and degeneration discernible, but there was also ample regeneration and repair going on at the same time. The lytic changes seen between 2–6 hr. had further progressed with formation of a large empty space in the dermis. These changes were associated with exudation around vascular structures. As in the 24 hr. sections, fibrin was seen as thrombi in the lumen of vessels, in vessel walls, in the adventitia and in paravascular connective tissue (Fig. 12). An interesting aspect of muscle injury was the invasion of degenerating myofibres by macrophages. This proceeded in some muscle fibres to the point where a shell of the degenerating myoplasm and myofibrils surrounded a nest of macrophages. As in mechanical and allergic injury (More and Movat, 1959), the disintegrating myofibres were intermingled with blue-staining AMP material derived from the surrounding perimysium. This appeared to be a process similar to the above described disintegration of fat cells.

Reparative changes were taking place from the periphery of the lesion. Fibroblasts and macrophages were numerous at these sites. The fibroblasts were situated between broken-up and widely separated collagen fibres. Many of these cells were surrounded by an AMP-containing substance. The latter was condensed around some of these cells and appeared either as granular or as a delicate fibrillar network extending into the spaces between the collagen fibres.

Five days.—The empty space described in the previous sections was enlarged due to an almost complete lysis of the central abscess-like area. This space was lined partly by a fibrinous band, resembling that described in mechanical injury. The proliferation of fibroblasts with deposit of an AMP-rich young tissue had further progressed to the formation of a granulation tissue. The latter was composed almost exclusively of numerous proliferating fibroblasts with AB-positive delicate fibres between them. A few inflammatory cells and remnants of broken-up collagen fibres were interspersed in this tissue. The collagen fibres, however, had undergone a certain orientation along with the new delicate blue fibres. Fibrinoid was present in the wall and in the vicinity of several vessels (Fig. 13). It was homogeneous, acidophilic and refractile. It lay between the newly formed AB-positive fibres having a wavy shape.

Eight days.—The lysis of the abscess seen in the 5-day sections was missing in this animal. The granulation tissue around the abscess was similar to the previous one. It was more abundant and more dense, especially at the periphery.

Fourteen days.—The dermis was extremely thick and made up entirely of granulation tissue. This had the appearance of a tissue culture of fibroblasts. The spaces between the fibroblasts were filled with metachromatic material (Fig. 14). In Pentachrome II, this metachromatic material stained blue. It

was fibrillar and vacuolated. In many areas very delicate faint red collagen fibres could be seen between the proliferating fibroblasts. The majority of the proliferating cells, in the areas where the tissue was looser, were oriented around vessels. In parts where the tissue was dense the cells had a linear arrangement. A small number of pseudoeosinophils, polymorphs and macrophages was still present in the granulation tissue. No plasma cells were encountered.

Experiment III. Physical injury (radiation)

Fourteen days.—The dermis appeared normal. A cellular reaction was present in a rather well circumscribed area in the loose connective tissue of the subcutis bordering the striated muscle layer of the skin. The cells were histiocytes and pseudoeosinophils. The interstitial connective tissue in these areas did not show any changes.

Eighteen days.—The pathological changes were in the same areas as those seen at 14 days. A large artery showed complete necrosis and disintegration of its wall, and sero-fibrinous exudate extended from the vessel along the tissue spaces in various directions (Fig. 15). The exudate separated the collagen fibres from one another in the loose connective tissue of the subcutis. In the Trichrome, delicate threads of fibrin were seen to surround collagen fibres and in areas where these fibres were more widely separated, abundant exudate had been precipitated (Fig. 16). By being situated along and in between the collagen fibres and proliferating cells, the exudate had the appearance of more or less homogeneous wavy acidophilic bands, especially in areas where it appeared more condensed. However, delicate threads of red-staining fibrin were seen along and between individual collagen fibres, some forming curly and knotty structures. The proliferating cells were histiocytes and fibroblasts, but a fair number of pseudoeosinophils had infiltrated the damaged area. Some of these cells, especially the fibroblasts, were quite atypical, reaching at times the size of a giant cell.

The injury to the collagen was not uniform. In some parts it consisted only of slight to moderate swelling, while in other areas the swelling was more marked, the colouration with collagen stains was diminished, and occasionally fragmentation and disintegration were noted (Fig. 17, 18).

Thirty days.—An exudate composed of granular protein material, histiocytes and pseudocosinophils was present in a circumscribed area. The granular protein material stained in most parts with acid dyes, but it stained partly with the AMP stains. Increased homogeneous AMP was present between separated collagen fibres. Damage to collagen was present as in the 18-day sections. No signs of repair were noted. No plasma cells were present at any stage studied.

DISCUSSION

Fibrinoid as a derivative of fibrinous exudate could be observed in all three types of injury. However, a " fibrinoid degeneration of collagen " was not seen. In mechanical injury one could trace the gradual development of fibrinoid. In early lesions fibrinous exudate covered the cut edge of the incision (Fig. 2, 3 and 4). When organization was fairly advanced there were clumps and bands of acidophilic fibrinoid in the young cellular and mucopolysaccharide-rich connective tissue (Fig. 5 and 6). A similar process was observed also in the lesion developing after injury by trypsin (Fig. 12 and 13) and radiation (Fig. 15 and 16).

In mechanical injury fibrinoid develops following outpouring of fibrin from severed vessels. It is not, as frequently quoted (Klemperer, 1950), a conversion or degeneration of collagenous fibres or of ground substance into fibrinoid (Wu, 1937). In chemical and radiation injury the fibrin is exuded from damaged blood vessels. It seems to develop after injury, presumably depolymerization, of the ground or cementing substances, which is followed by increased tissue and vascular permeability. In all these instances, where the injury is not continuous and repair is not inhibited, the exudate is rapidly absorbed. Fibrinoid is nonspecific and it can develop in a number of heterogenous conditions (Movat, 1958 and Movat and More, 1957).

In the early phases of exudation the acid mucopolysaccharides normally present in the dermis were diminished, or not demonstrable at all, as if " diluted " or " lysed " by the exudate. Later when organization set in, an increase of mucopolysaccharides occurred hand-in-hand with proliferation of fibroblasts and formation of young capillaries (Fig. 6, 7, 13 and 14). Early collagenization could be observed in mechanical injury. Thus the first step in organization was formation of a young mucopolysaccharide-rich connective tissue. It would appear that the protein-rich exudate furnished the matrix from which the AMP-rich young connective tissue developed, and this in turn, the matrix from which collagenous fibres arose.

One of the objectives of these experiments was to determine whether plasma cells appear as a reaction following mechanical, chemical or radiation injury. In these experiments plasma cells did not appear in association with injury of connective tissue and formation of fibrinoid. In connective tissue injury of this duration produced by injection of antigen, abundant plasma cells were always present (More and Movat, 1959 and Movat, 1956). This fact favours our view that plasma cells in tissue indicate local antibody production (More and Movat, 1958, 1959*a* and *b* ; Movat, 1956 and Movat and More, 1955). The fact that in man plasma cells are seen in late phases of radiation injury may be due either to infection or the presence of autoantigens formed in the damaged tissue with the passage of time.

SUMMARY

Mechanical injury (incision) of the rabbit's skin is followed by haemorrhage into the incision. Fibrin is precipitated along the cut edges and in the depth of the incision. The conglomeration and inspissation of plasma. but particularly fibrin, results in the formation of typical fibrinoid. A " fibrinoid degeneration of collagen " does not occur under these circumstances. The exudate furnishes the matrix for the formation of a young, acid mucopolysaccharide-rich connective tissue, which later is converted into a collagenous scar.

After chemical injury of the rabbit's skin by trypsin, degeneration and necrosis of connective tissue occur accompanied by an acute (polymorphonuclear) inflammatory reaction. A concomitant fibrinous exudation is found in the wall of a large number of vessels and in the surrounding connective tissue. The unorganized remnants of fibrin found in this tissue have the morphological and tinctorial characteristics of fibrinoid. Fibrinoid in trypsin injury does not derive from ground substance or collagen, but from an inspissation of exuded plasma, predominantly fibrin. The increased acid mucopolysaccharides in areas of damage and fibrin deposition represent early regeneration and repair of connective tissue.

The changes after physical (radiation) injury are in part also a fibrinous exudate. Fibrinoid in this instance also develops from exudation and inspissation of the exuded plasma proteins. Vascular injury seems to be the causative factor in this exudation. Damage to collagen and ground substance is manifested as a swelling and disintegration, but no "fibrinoid degeneration" of connective tissue takes place.

Fibrinoid can develop under various unrelated conditions and is therefore non-specific.

A plasma cellular reaction does not occur in the lesion which follows non-specific, *i.e.*, mechanical, chemical or physical injury of connective tissue. This absence of plasma cells gives further support to the hypothesis that plasma cells appear only in association with antigenic stimulation.

The authors wish to thank Miss Käthe Oellerman for technical assistance and Mr. P. H. Mott for taking the photomicrographs.

REFERENCES

KLEMPERER, P.—(1950) *Amer. J. Path.*, **26**, 505.

MORE, R. H. AND MOVAT, H. Z.—(1958) *J. Path. Bact.*, **74**, 127.—(1959*a*) *Lab. Invest.*, **8**, 873.—(1959*b*) *Arch. Path. (Lab. Med.)*, **67**, 679.

MOVAT, H. Z.—(1956) *Beitr. Path. Anat.*, **116**, 239.—(1958) *Amer. J. med. Sci.*, **236**, 373.

Idem AND MORE, R. H.—(1955) First Canadian Conference for Research into Rheumatic Diseases, 65.—(1957) *Amer. J. clin. Path.*, **28**, 331.

WU, T. T.—(1937) *Virchows Arch.*, **300**, 373.

THE PRODUCTION OF HIGH TITRE POLIOVIRUS IN CONCENTRATED SUSPENSIONS OF TISSUE CULTURE CELLS

J. C. N. WESTWOOD, G. APPLEYARD, D. TAYLOR-ROBINSON AND H. T. ZWARTOUW

From the Microbiological Research Establishment, Porton, Salisbury, Wilts.

Received for publication July 29, 1959

"MINIMUM requirements", as laid down by the Ministry of Health for material to be used in the manufacture of poliomyelitis vaccine, demand a virus infectivity titre of at least 10^6/ml. Though the material used in practice normally exceeds this titre by about ten-fold, it seems likely that the borderline efficiency of the vaccine, and indeed of inactivated virus vaccines in general, is due to their low content of virus antigens. While high titre virus suspensions may be produced by physical or chemical concentration procedures, such methods entail unavoidable losses in virus and, at least with the smaller viruses, are too cumbersome for use on a large scale. In this laboratory, the possibility is being explored of producing high concentrations of poliovirus directly into the supernatant fluid of tissue cultures by the infection of concentrated suspensions of cells. The work reported here shows that the method is effective and simple.

Published results indicate that the average yield of poliovirus from single cells of serially cultivated cell lines lies between 100 and 500 infective units. (Girardi, McMichael and Henle, 1956; Darnell and Eagle, 1958; Dunnebacke and Reaume, 1958; Roizman, Hopken and Mayer, 1958). If this yield cannot be exceeded, an increase in the final virus concentration can be attained only by increasing the number of infected cells per unit volume of fluid. Churcher, Sheffield and Smith (1959) achieved this in monolayer cultures by growing cells on all six sides of hexagonal Pyrex bottles and infecting them in half the normal volume of medium. This method cannot, however, be easily adapted to large scale production whereas cell suspensions may be used at any desired volume and any desired concentration.

Pereira (1954) successfully infected suspensions of chick embryo cells with fowl plague virus and McLimans, Giardinello, Davis, Thomas and Kucera (1957) showed that the cells of the ERK1 cell line (Westwood, Macpherson and Titmuss 1957) would support the multiplication of poliovirus when grown and infected in suspension. Girardi *et al.* (1956), investigating the multiplication of polio and influenza viruses in suspensions of HeLa cells found that cell survival was optimal at a concentration of 5×10^5/ml. and concluded that, above this figure, exhaustion of essential nutrients led to early cell deterioration. This concentration of cells gave yields of about 100 plaque-forming units (PFU) per cell of poliovirus, but would clearly be inadequate for the production of virus in high titre. It seemed necessary therefore to investigate first the factors governing cell survival under the adverse conditions obtaining in high cell concentrations.

The work was originally undertaken, in parallel with that already reported from University College Hospital (Churcher *et al.* 1959) in order to investigate

methods of producing flocculating poliovirus antigen (Smith, Sheffield, Lee and Churcher, 1956) without the necessity for centrifugal concentration.

MATERIALS AND METHODS

Poliovirus.—Type 1 (Brunhilde) poliovirus, obtained through the courtesy of Professor Wilson Smith, was used throughout.

Tissue cell line.—The ERK1 cell line, derived from embryo rabbit kidney (Westwood *et al.* 1957) was used. For the sake of continuity with previous work at U.C.H., the "D" line of cells, originally sent to U.C.H. in February, 1957, was reintroduced into this laboratory in January, 1958, and was used for the present investigations.

Growth and preparation of cells.—Cells were grown in stationary Roux bottle cultures for 7 days without change of medium and were just confluent, without overcrowding, at the end of this time.

The growth medium was a modification of the high glucose, high bicarbonate medium described by Zwartouw and Westwood (1958) and consisted of:—calf serum, Seitz-filtered and inactivated at 60° for 120 min., 15 per cent; tryptic digest of lean beef, 5 per cent; yeast extract, 0·5 per cent; Fildes' peptic digest of sheeps' blood, 0·1 per cent; Earle's saline, modified to contain 4 g./l. of glucose and 3 g./l. of sodium bicarbonate, to 100 per cent.

Neomycin, 125 μg./ml.; Penicillin, 125 units/ml. and Streptomycin, 125 μg./ml. were included in the medium.

Cells were harvested with a mixture of 0·05 per cent trypsin (DIFCO 1/250) and 0·05 per cent EDTA (Ethylene-diamine-tetra-acetic acid, disodium salt; British Drug Houses Ltd.) in phosphate-buffered saline, pH 7·2, modified by the addition of sodium bicarbonate to a concentration of 0·1 per cent. After centrifugation from the trypsin-EDTA mixture the cells were resuspended in a small volume of medium, counted in a haemocytometer and diluted to the desired concentration.

Growth of poliovirus.—Cell suspensions were distributed into 250 ml. Pyrex feeding bottles, and virus inoculum added to bring the final volume in each bottle to 10 ml. Bottles were gassed with air containing the appropriate concentration of CO_2, stoppered and placed in a slow roller drum at 37°. After suitable time intervals, the cultures were cooled, cell débris removed by centrifugation at 2000 **g.** for 5 min., and the supernatant fluids stored at 2–4°. Samples were titrated within one week of harvesting.

The medium used for poliovirus replication was the same as that used for cell growth except that it contained only 10 per cent of calf serum and the glucose and bicarbonate concentrations were varied as described in the text.

Poliovirus titrations.—Infectivity titrations were carried out by Dulbecco's plaque technique as modified by Cooper (1955) and titres are reported as plaque-forming units (PFU) per ml.

Flocculation titrations.—Specific rabbit antisera, kindly supplied by Professor Wilson Smith, were absorbed to remove ERK1-cell precipitins and used in the micro-flocculation test described by Smith, Sheffield, Churcher and Lee (1956) to measure the flocculating potency of poliovirus preparations. Antigen dilutions were titrated against a fixed serum dilution, chosen to give optimal flocculation for the particular batch of serum. Antigen potency is reported as the highest initial dilution of antigen giving a positive reaction.

RESULTS

Maintenance of Viability in Cell Suspensions

It was assumed that the virus yield from cells infected in suspensions was likely to depend on the success with which cell viability was maintained. For the technique to be successful virus titres of at least 10^9 PFU per ml. should be obtainable. This would require cell concentrations of $5–10 \times 10^6$/ml. provided that the expected yield of virus per cell was attained. Experiments were therefore performed to determine the optimal conditions for cell survival at concentrations of this order. The two most obvious factors to be considered were the

rapidity with which glucose would be exhausted and the rate at which the pH would fall below tolerable limits.

In an experiment relating changes in pH, glucose exhaustion and cell viability, 3 cell concentrations and 2 levels of bicarbonate-CO_2 buffer were examined. From each set of replicate cultures in the roller drum at 37°, a sample bottle was withdrawn for assay at various time intervals. pH determinations were made at 37°, using an enclosed glass electrode, within thirty seconds of each bottle being opened. Exhaustion of glucose from the medium was detected by a colorimetric test using "Clinistix" (Ames Co., London). The medium contained initially 4 g./l. of glucose, the highest concentration found by Zwartouw and Westwood (1958) to be non-toxic, and a fall to 0·5 g./l. was taken to indicate imminent exhaustion. Positive staining with trypan blue was assumed to indicate loss of cell viability (Girardi *et al.* 1956, McLimans, Davis, Glover and Rake, 1958).

TABLE I.—*The Relationship of pH Fall and Glucose Utilization to Maintenance of Cell Viability at Various Cell Concentrations*

Buffer concentration	Incubation time in hr.	Cell concentration/ml. 5×10^6 pH	Glucose*	Per cent dead cells	10×10^6 pH	Glucose	Per cent dead cells	20×10^6 pH	Glucose	Per cent dead cells
$NaHCO_3$: 2 g/l. CO_2: 5 per cent.	0	7·65	++	3·5	7·65	++	3·5	7·65	++	3·5
	2	7·43	++	..	7·22	++	..	6·7	++	..
	5	7·3	++	21	6·99	++	23	6·27	++	29
	10	7·0	++	19	6·75	+	17	6·65	+	27
	24	6·7	+	37	6·75	−	35	6·8	−	52
$NaHCO_3$: 4 g/l. CO_2: 10 per cent.	0	7·54	++	3·5	7·54	++	3·5	7·54	++	3·5
	2	7·42	++	..	7·3	++	..	6·98	++	..
	5	7·36	++	18	7·18	++	17	6·9	−	23
	10	7·2	++	18	6·95	+	25	6·9	−	34
	24	7·0	−	40	7·1	−	53	7·08	−	69

* Glucose. ++ = > 1 g/l. + = 1·0 − 0·5 g/l. − = < 0·5 g/l.

The results (Table I) showed the better pH control obtained by doubling the normal buffer concentration and also indicated the rapidity with which glucose was exhausted at high cell concentrations when a rapid fall in pH was prevented. In other experiments carried out on both infected and uninfected cultures it was found that higher concentrations of glucose were tolerated provided that the buffer concentration was increased to compensate for the extra acid production. However, even when 8g./ l. of glucose was initially present, all glucose was exhausted within 16 hr. by cells at a concentration of 10^7/ml. The results shown in Table I suggest that the rate of cell death is not directly correlated with fall in pH and this conclusion was confirmed in other experiments. The fact that more cells died in those suspensions in which the pH was prevented from falling below 6·9 suggests rather that glucose exhaustion may be the more important factor. However the divergence in the rate of cell death occurred after 10 hr., at which time three quarters of the cells were still not stainable with trypan blue and glucose was still demonstrable in the cultures containing 10^7 cells per ml.

It was concluded that, at a concentration of 10^7/ml., about three-quarters of the cells could be maintained in a viable state for a ten hour period without exhaustion of glucose. Since it seemed probable that adequate control of the rate of fall of pH would favour optimal virus production, a medium containing 4g./1. of both glucose and sodium bicarbonate was adopted.

Virus Production from Concentrated Cell Suspensions

Virus inoculum

It was clear from the results of the experiments already discussed that virus replication must be restricted to a single cycle. The ten hour period during which cell viability could be maintained at a high level should then suffice for maximum virus replication. This conclusion was confirmed by experiments in which the multiplicity of infection was varied in cell suspensions containing 10^7 cells per ml. Table II shows the result of one such experiment.

TABLE II.—*Virus Yields at Various Multiplicities of Infection*

Inoculum PFU/cell	Virus titre at 24 hr. $\times 10^{-9}$	Virus yield PFU/cell
0·2	1·35	135
1·0	1·65	165
2·0	2·35	235
20·0	2·30	230

Cell concentration 10^7/ml.

Increasing the multiplicity of infection above 2 PFU per cell, at which level about 86 per cent of the cells should in theory be initially infected, did not improve the virus yield. This multiplicity was therefore adopted for all future experiments.

Effect of cell concentration on virus yield

In a number of experiments it was found that increasing virus titres were obtained as the cell concentration was increased up to 10^7 cells per ml. but at or above the 2×10^7 concentration an abrupt fall in virus yield occurred (Table III). From Table I it can be seen that control of the rate of fall of pH begins to fail at this level, even with the higher buffer concentration. That pH is in fact a contributory factor in determining the optimal cell concentration under these experimental conditions is suggested by Experiment 2 of Table III, in which two levels of buffer were compared. The effect of the better pH control at the higher buffer concentration is clearly shown. It may also be seen that, at both buffer levels, the highest virus yield in terms of PFU per cell occurred at the next lower cell concentration to that giving the highest virus titre.

Despite the poorer yield per cell, a concentration of 10^7 cells per ml. was selected for further work because it produced the highest virus titres.

Effect of pH

Investigation of the effect of pH on virus yield is complicated by the fact that the pH cannot be maintained at a constant value under the conditions of these experiments. From experiments in which the pH was followed under

TABLE III.—*Effect of Cell Concentration on Virus Yield*

Expt.	Buffer concentration	Cell concentration/ml. × 10^{-6}	Virus titre × 10^{-8}	Virus yield PFU/cell
1	$NaHCO_3$: 4 g./l. CO_2 : 10 per cent	2·5	0·45	18
		10	10	100
		20	7	35
		30	0.5	—
		40	0·5	—
2 A	$NaHCO_3$: 2 g./l. CO_2 : 5 per cent	2·5	8	320
		5	11·5	230
		10	7·1	71
		20	0·05	0·25
		40	0·05	0·1
2 B	$NaHCO_3$: 4 g./l. CO_2 : 10 per cent	2·5	2·8	112
		5	10.5	210
		10	13	130
		20	10	50
		40	0·22	0·6

various medium conditions, it was possible to conclude that the best yields were obtained only when the pH was not permitted to exceed the limits of 7·5–6·8 during the course of the experiment. The optimal pH range for poliovirus production cannot, however, be determined in the presence of glucose without continuous pH regulation. This problem is being further investigated.

Effect of time of incubation on infectivity titre

The intracellular development of infective virus was followed in one experiment only. The rate of appearance of virus was similar to that observed in monolayer cultures (Roizman *et al.*, 1958) and maximum titre was observed at 12 hr. The time relationships of virus release have been more closely followed and Table IV shows the results of three experiments in which sample cultures were harvested at the stated time intervals after infection. In all such experiments the highest infectivity titre in the culture fluid was obtained at 16–24 hr.

TABLE IV.—*Effect of Time of Incubation on Infectivity and Flocculation Titres*

Expt.	Hours after infection	Virus titre × 10^{-8}	Virus yield PFU/cell	Reciprocal of flocculation titre
1	8	<5	—	8
	16	23	230	16
	24	32·5	325	16
	48	16	160	16
	72	2·4	24	16
2	8	6·8	68	8
	16	28	280	16
	24	15	150	16
	48	26	260	16
	72	<0·1	—	16
3	8	<0·1	—	1
	16	16	160	8
	24	20	200	8
	48	4·5	45	16

A terminal fall in titre of about 1 log unit, due presumably to virus inactivation, was evident at either 48 or 72 hr. It was found that virus suspensions set up under the test conditions but in the absence of cells lost about 90 per cent of their infectivity titres within 48 hr.

Flocculating Activity of Virus Preparations

Since the original purpose of this work was to develop a method for producing flocculating antigen without the necessity for virus concentration, many of the preparations were titrated for flocculating potency as well as infectivity. The experiments presented in Table IV show that high infectivity titres in the supernatants of early harvests, up to 24 hr., are associated with satisfactory flocculating activity. It is also evident from this table that flocculating activity is more stable than infectivity at 37°. The antisera used in the flocculation tests (Belyavin, personal communication) contained antibodies against both the D and C components of the virus (Mayer, Rapp, Roizman, Klein, Cowan, Lukens, Schwerdt, Schaffer and Charney, 1957) and we have made no attempt to distinguish between the two activities. It is known (Roizman, Mayer and Roane, 1959) that D activity may be converted to C as a result of heat treatment, and it it possible that the retention of flocculating activity, despite loss of infectivity after 24 hr., may be due to this change. Although this would seem unlikely at the temperature and times involved, it was decided to harvest virus for flocculating antigen during the 16–24 hr. period when both infectivity and flocculating activity had reached their peak, so as to avoid the possibility of degradation of D component. Flocculation titres of between $\frac{1}{16}$ and $\frac{1}{32}$ are regularly obtained in this manner.

DISCUSSION

The experiments quoted in this paper show that high titre virus preparations may be obtained by infecting cell suspensions containing 10^7 cells per ml. The virus yields, in terms of PFU per cell, are comparable to those obtained in monolayer cultures. The work reported has been confined to Type 1 poliovirus and to the use of a fixed volume of 10 ml. of suspension agitated by rolling in a 250 ml. feeding bottle. The medium used was complex and contained a high proportion of serum. These conditions were imposed as a matter of convenience for early experimental investigations and subsequent work have shown that the method is applicable to the other poliovirus types, that larger volumes of suspension may be used if conditions are suitably adjusted and that similar virus yields may be obtained in a simple physiological salt solution in the absence of serum, nitrogenous nutrients and glucose. This work, which will be reported in detail, shows that the theoretical advantages of the method should be fully realisable.

The practical limitation to the use of this technique on a production scale is likely to be the supply of cells, but recent work (Cooper, Burt and Wilson, 1958) suggests that this problem may be solved by the growth of cells in suspension on the chemostat principle.

SUMMARY

The possibility has been investigated of obtaining high titre poliovirus preparations by infecting suspended tissue culture cells at high concentration in order to obtain the released virus in the smallest practicable volume of fluid.

It was found that three-quarters of the cells in a suspension containing 10^7 cells per ml. could be maintained in a viable state as judged by absence of staining with trypan blue, for a period long enough to allow a single cycle of virus replication.

If a multiplicity of infection of 2 PFU per cell or higher were used, maximum virus titres in the culture fluid were reached within 24 hr. and virus yields of up to 325 PFU per cell were obtained. Poliovirus preparations containing over 10^9 PFU per ml. could be obtained in this way.

REFERENCES

Churcher, G. M., Sheffield, F. W. and Smith, W.—(1959) *Brit. J. exp. Path.*, **40**, 87.

Cooper, P. D.—(1955) *Virology*, **1**, 397.

Idem, Burt, A. M. and Wilson, J. N.—(1958) *Nature, Lond.*, **182**, 1508.

Darnell, J. E. and Eagle, H.—(1958) *Virology*, **6**, 556.

Dunnebacke, T. H. and Reaume, M. B.—(1958) *Ibid.*, **6**, 8.

Girardi, A. J., McMichael, H. and Henle, W.—(1956) *Ibid.*, **2**, 532.

McLimans, W. F., Davis, E. V., Glover, F. L. and Rake, G. W.—(1958) *J. Immunol.*, **79**, 428.

Idem, Giardinello, F. E., Davis, E. V., Thomas, W. J. and Kucera, C. J.—(1957) Fourth International Poliomyelitis Conference, Geneva, Switzerland.

Mayer, M. M., Rapp, H. J., Roizman, B., Klein, S. W., Cowan, K. M., Lukens, D., Schwerdt, C. E., Schaffer, F. L. and Charney, J.—(1957). *J. Immunol.*, **78**, 435.

Pereira, H. G.—(1954) *J. gen. Microbiol.*, **10**, 500.

Roizman, B., Hopken, W. and Mayer, M. M.—(1958) *J. Immunol.*, **80**, 386.

Idem, Mayer, M. M. and Roane, P. R.—(1959) *Ibid.* **82**, 19.

Smith, W., Sheffield, F. W., Lee, L. H. and Churcher, G.—(1956) *Lancet*, i, 710.

Idem, Sheffield, F. W., Churcher, G. and Lee, L. H.—(1956) *Ibid.*, ii, 163.

Westwood, J. C. N., Macpherson, I. A. and Titmuss, D. H. J.—(1957) *Brit. J. exp. Path.*, **38**, 138.

Zwartouw, H. and Westwood, J. C. N.—(1958) *Ibid.*, **39**, 529.

REMOVAL OF CHOLESTEROL FROM RETICULO-ENDOTHELIAL CELLS*

A. J. DAY†

From the Sir William Dunn School of Pathology, University of Oxford

Received for publication August 10, 1959

WHEN finely divided suspensions of cholesterol or cholesteryl oleate or emulsions of triglycerides are injected intraperitoneally into rats, they are absorbed via the diaphragmatic lymphatics and are subsequently retained in the reticulo-endothelial cells of the sternal lymph nodes (French and Morris, 1960). When cholesteryl stearate or cholesteryl oleate suspensions are taken up, Sudan-staining material persists in the nodes for at least 12 months. On the other hand, when cholesterol or cholesteryl acetate suspensions are taken up only traces of sudan staining material remains after 2 months (Day and French, unpublished). In the experiments described in this paper the rate of removal of cholesterol and cholesteryl esters, taken up by the nodes in various forms has been investigated chemically. The changes in total fatty acid and phospholipid content, which occur in the nodes following the uptake of cholesterol or cholesteryl oleate, have also been determined.

MATERIALS AND METHODS

Lipid preparations for intraperitoneal injection

Aqueous suspensions of cholesterol, cholesteryl oleate and cholesteryl stearate were prepared by slowly adding, with constant stirring, a warm solution of 1·0 g. of cholesterol or the ester in acetone to 400 ml. of boiling water. The suspension obtained was concentrated and filtered through glass wool. It was mixed with equal parts of a solution containing 0·5 per cent Tween 60 in 5 per cent glucose and bovine albumin (Armour) was added to give a final preparation containing 4 mg./ml. of cholesterol in 2·5 per cent albumin. Each animal had 2 ml. of this preparation administered intraperitoneally. Chromatographic analysis as described below was carried out to determine the free and ester cholesterol content of these preparations. No detectable ester was present in the cholesterol suspension nor any free cholesterol in the cholesteryl stearate suspension. The cholesteryl oleate suspension contained 10 per cent free cholesterol.

When changes in the fatty acid and phospholipid content of the nodes were investigated, Tween 60 and glucose were omitted from the suspensions. Chemical analysis as described below was carried out on these suspensions to establish that they did not contain any phospholipid or any triglyceride esterified fatty acid.

Rabbit lipoprotein.—Serum from rabbits fed for 2–3 months on a diet rich in cholesterol was used as a source of lipoprotein. The cholesterol content of the serum was determined and adjusted to 10 mg./ml. by addition of 0·9 per cent sodium chloride, 2 ml. were injected intraperitoneally.

Cholesterol in corn oil.—An emulsion was prepared from corn oil in which 2·5 per cent cholesterol had been dissolved. It had the following composition : 0·25 per cent cholesterol, 10 per cent corn oil, 0·5 per cent Tween 60 and 5 per cent glucose. Emulsification was carried out by mixing for 10 min. in a Waring blender. The dose was 2 ml. injected intraperitoneally.

* This work was aided by a personal grant from the Medical Research Council.

† Present address : Department of Human Physiology and Pharmacology, University of Adelaide, South Australia.

Experimental procedure

In order to compare the rates of removal of cholesterol from the nodes, groups of male albino rats (200 g.) were injected with each of the different preparations. Eight rats from each group were killed at intervals of 24 hr., 4, 10 and in some cases 28 days after injection. The sternal lymph nodes were removed and the cholesterol content (ester and free) was determined.

To investigate the changes in phospholipid and fatty acid content of the nodes 56 rats were divided randomly into 7 groups of 8 animals. One group was untreated ; 3 groups were injected with a cholesterol suspension and 3 groups with a cholesteryl oleate suspension. Groups of animals were killed 24 hr., 4 and 10 days after the injections and the total cholesterol, total (esterified) fatty acid and phospholipid content of the sternal lymph nodes was determined.

Lipid determinations

Animals were killed by ether anaesthesia ; the sternal nodes were removed and the lipids extracted as previously described (Day and French, 1959). The ester and free cholesterol were separated chromatographically (Kerr and Bauld, 1953) and determined by the method of Zlatkis, Zak and Boyle (1953). For total cholesterol, total (esterified) fatty acid and phospholipid determinations a 3 : 1 ethanol : ether extract was prepared. The total (esterified) fatty acid was determined as described by Stern and Shapiro (1953). This method does not measure cholesteryl ester fatty acids. Phospholipid was determined as described by Allen (1940). Total cholesterol was determined directly by the method of Zlatkis *et al.* (1953).

RESULTS

Removal of cholesterol from the sternal nodes

The mean ester and free cholesterol content at intervals after the administration of the various preparations of cholesterol are shown in Table I. Fig. 1 shows the total cholesterol in the nodes, at various intervals after uptake, expressed as a percentage of that present 24 hr. after injection. When free cholesterol suspension or cholesteryl oleate suspension was taken up the level had fallen appreciably by 4 days and by 10 days only 10–15 per cent of the excess cholesterol remained. (Control nodes contain approximately 150 μg. cholesterol.) No difference in removal rate was apparent between the 2 suspensions.

TABLE I.—*Micrograms of Cholesterol (Ester or Free) Remaining in Rat Sternal Lymph Nodes at Intervals Following the Intraperitoneal Injection of Various Preparations of Cholesterol*

(Mean of 8 determinations with the estimated standard error)

Preparation injected	Cholesterol fraction	24 hr.	4 days	10 days	28 days
Cholesterol suspension	Ester	107±9·6	114±13·1	10±2·9	—
	Free	577±55·0	234±16·3	192±16·4	—
Cholesteryl oleate suspension	Ester	543±41·1	214±27·9	35±6·2	0
	Free	462±35·3	320±26·8	244±21·0	161±12·8
Cholesterol in corn oil emulsion	Ester	142±15·3	68±8·7	—	0
	Free	428±27·8	186±12·3	—	186±11·8
*Cholesteryl stearate suspension	Ester	368±37·4	843±69·0	339±71·0	—
	Free	302±64·5	575±34·0	293±22·2	—
Rabbit lipoprotein	Ester	203±33·5	112±15·5	22±2·3†	—
	Free	255±14·1	250±17·2	179±9·2	—

* Four rats only.
† Six rats only.

Cholesterol emulsified in corn oil appeared to be removed more rapidly initially, than the other preparations. Four days after its uptake there was less than half of the excess cholesterol in the nodes (Table II). In this experiment, no observations were made at 10 days but at 28 days the nodes still contained a slight excess of cholesterol.

Cholesteryl stearate apparently behaved differently. The suspension was more unstable and less readily absorbed from the peritoneal cavity. At 24 hr., some

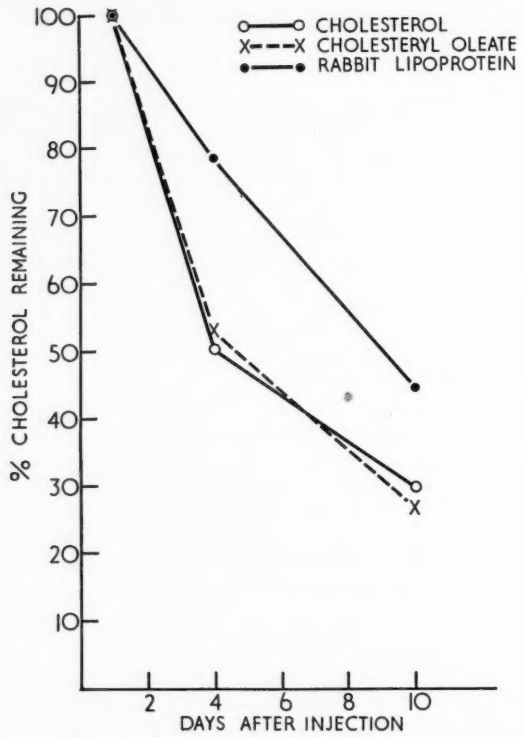


FIG. 1.—Removal of cholesterol from rat sternal lymph nodes following the uptake of various preparations of cholesterol. The cholesterol present in the nodes at intervals after injection is expressed as the percentage of that present at 24 hr.

TABLE II.—*Total Cholesterol Remaining in Rat Sternal Lymph Nodes* 4 *and* 10 *Days after the Intraperitoneal Injection of Cholesterol or Cholesteryl Esters*

Preparation injected	4 days	10 days	
	(*a*)	(*a*)	(*b*)
Cholesterol suspension	50·9	29·5	58·0
Cholesteryl oleate suspension	53·1	26·6	52·2
Cholesterol in corn oil emulsion	44·6	—	—
Cholesteryl stearate suspension	212·0	94·4	44·5
Rabbit lipoprotein	79·0	43·8	55·5

(Expressed as a percentage of the amount present at (*a*) 24 hr., (*b*) 4 days)

flocculation occurred in the peritoneal cavity and the diaphragmatic and retrosternal lymphatics were filled with precipitated material. This appearance of the lymphatics was still seen, but to a lesser degree, at 4 days. Because of this delayed absorption the maximum level of cholesteryl stearate was not attained in the nodes until 4 days after injection. The percentage rate of removal from 4–10 days, however, was as rapid as with cholesterol or cholesteryl oleate (Table II).

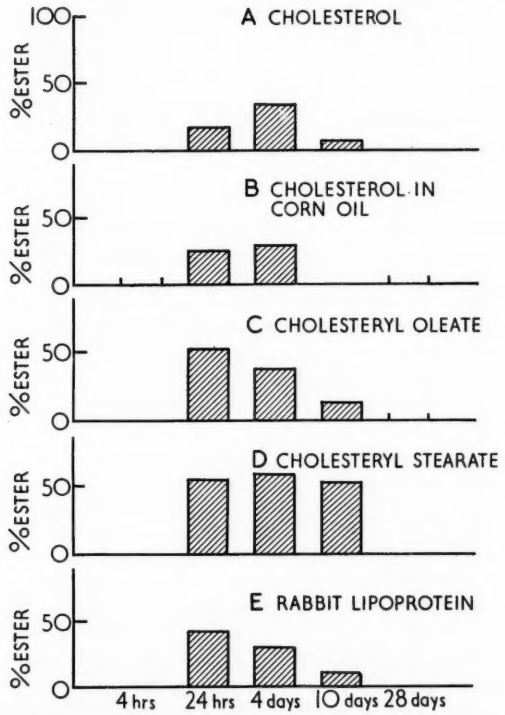


FIG. 2.—Percentage of the total cholesterol present as ester in sternal lymph nodes at intervals following the intraperitoneal injection of various preparations of cholesterol.

In the rats injected with serum from cholesterol-fed rabbits there was appreciable uptake of cholesterol by the nodes. The absorption from the peritoneal cavity was also slow, and material was still present in the retrosternal and diaphragmatic lymphatics 24 hr. after injection. The relatively high level at 4 days was therefore not an indication of any slower removal of cholesterol from the nodes. The removal rate from 4–10 days was similar to that for the other preparations.

Changes in free and ester cholesterol

The percentage ester present in the nodes at intervals after the administration of the various preparations is shown in Fig. 2. Control nodes contain approximately

150 μg. free cholesterol but no ester cholesterol. When free cholesterol was taken up, ester appeared in the nodes and its percentage rose. When cholesteryl oleate or rabbit lipoprotein (containing 73·3 per cent cholesteryl ester) was taken up the free cholesterol content of the nodes increased (Table I) and the initial high percentage ester fell, indicating that hydrolysis was occurring.

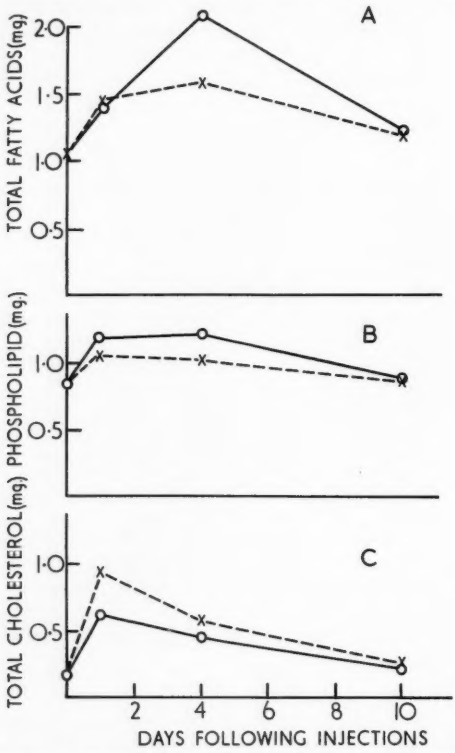


FIG. 3.—The accumulation of cholesterol, phospholipid and total (esterified) fatty acid in sternal lymph nodes following the uptake of cholesterol or cholesteryl oleate.

○ ——— ○ cholesterol.
×- - - - - - - - - × cholesteryl oleate.

When cholesteryl stearate was taken up, the continued uptake of ester by the nodes for the 4 days after injection offset the rise in free cholesterol which occurred in the nodes. Although the percentage ester did not fall it was apparent from the increased free cholesterol content of the nodes (Table I) that hydrolysis of cholesteryl stearate had occurred.

Changes in total fatty acid and phospholipid content

Table III and Fig. 3 show the changes which occur in total fatty acid, phospholipid and cholesterol content of the sternal lymph nodes following the uptake of cholesterol or cholesteryl oleate suspensions.

TABLE III.—*Micrograms of Cholesterol, Total (Esterified) Fatty Acid and Phospholipid in the Sternal Lymph Nodes at Intervals Following the Intraperitoneal Injection of Cholesterol or Cholesteryl Oleate*

Preparation injected	Fraction	0 days	24 hr.	4 days	10 days
Control	Cholesterol	—*	—	—	—
	Total fatty acid	1030±96	—	—	—
	Phospholipid	840±61	—	—	—
Cholesterol	Cholesterol	—	630±117	462±48	233±11
	Total fatty acid	—	1370±142	2130±326	1230±71
	Phospholipid	—	1170±123	1200±126	880±25
Cholesteryl oleate	Cholesterol	—	945±93	589±43	252±16
	Total fatty acid	—	1410±88	1590±77	1170±72
	Phospholipid	—	1050±100	1010±29	860±10

(Mean of 8 determinations with the estimated standard error)

* Not determined.

The increase in cholesterol level in the nodes 24 hr. after the injection of cholesterol or cholesteryl oleate was accompanied by an increase in fatty acid and phospholipid. After 4 days the fatty acid and phospholipid content declined and at 10 days was only slightly above the level in the controls.

It will be noted from Fig. 3 that the increases in fatty acid and phospholipid were greater in the cholesterol treated group than in the cholesteryl oleate treated group, although cholesterol uptake was less in the former.

DISCUSSION

The removal of cholesterol from lymph nodes may be brought about in part by macrophages which become free and wander to other sites but, in addition, metabolic changes within the cells may facilitate removal. Esterification of cholesterol occurs in the lymph nodes and some may be removed in this form. Tompkins (1946), on histochemical evidence, has claimed that esterification of cholesterol by macrophages in the skin is an essential step in the removal of cholesterol in a soluble form as lipoprotein. When cholesterol was taken up initially in the form of various esters there was no evidence that it was removed any more rapidly than when taken up initially as unesterified cholesterol. However, this similarity in removal rates may be explained by the observation that hydrolysis of the esters also occurs in the nodes, so that after a few days the percentage ester is much the same whether free or ester cholesterol is taken up initially.

The accumulation of phospholipids and fatty acids in the nodes following the uptake of cholesterol may also be a part of the removal process. These substances may be derived from the blood or lymph or they may be synthesised within reticulo-endothelial cells in response to the presence of cholesterol. Zilversmit, Shore and Ackerman (1954) have shown that phospholipid, which accumulates in the aortae of rabbits fed cholesterol, is synthesised locally. They suggest that this facilitates the removal of cholesterol from the aorta as lipoprotein. Since the accumulation of lipid in the aorta in the cholesterol fed rabbit is largely within macrophages, the metabolic changes which they have described may occur within these cells.

The chemical analyses have shown that cholesterol is removed more rapidly from the nodes than had been indicated by the earlier histological observations

(French and Morris, 1960; Day and French, unpublished). It appears that, although some cholesterol persists in the nodes in trace amounts not detectable by the chemical method, much of the Sudan-staining observed at the later intervals could be due to the other lipids which were present in addition to these trace amounts of cholesterol.

SUMMARY

Suspensions of cholesterol and of various cholesteryl esters were injected intraperitoneally into rats and the uptake of these preparations in the sternal lymph nodes and their subsequent removal was observed. Almost all of the cholesterol taken up was removed from the nodes within 10 days, but there was no difference in the rate of removal of cholesterol with the different preparations.

The ingestion of unesterified cholesterol by the nodes was followed by an increase in cholesteryl ester, while the ingestion of cholesteryl esters was followed by an increase in free cholesterol. The uptake of cholesterol and cholesteryl oleate also resulted in the accumulation of phospholipids and total (esterified) fatty acids in the nodes. It was considered that these metabolic changes may influence the removal of cholesterol from reticulo-endothelial cells and the results were discussed from this standpoint.

I wish to acknowledge the continued interest of Professor Sir Howard Florey and the help and encouragement of Dr. J. E. French. I am grateful to Miss P. M. Harris for carrying out the phospholipid determinations and to Mr. G. J. Wren for technical assistance.

REFERENCES

Allen, R. J.—(1940) *Biochem. J.*, **34**, 858.
Day, A. J. and French, J. E.—(1959) *Quart. J. exp. Physiol.*, **44**, 239.
French, J. E. and Morris, B.—(1960) *J. Path. Bact.*, **79**, 11.
Kerr, L. M. and Bauld, W. S.—(1953) *Biochem. J.*, **55**, 872.
Stern, I. and Shapiro, B.—(1953) *J. clin. Path.*, **6**, 158.
Tompkins, E. H.—(1946) *Arch. Path.* (*Lab. Med.*), **42**, 299.
Zilversmit, D. B., Shore, M. L. and Ackerman, R. F.—(1954) *Circulation*, **9**, 581.
Zlatkis, A., Zak, B. and Boyle, A. J.—(1953) *J. Lab. clin. Med.*, **41**, 486.

SEQUENCE OF TISSUE, SERUM AND URINE CHANGES IN RATS TREATED WITH AMINONUCLEOSIDE

CORNELIA HOCH-LIGETI

From the Department of Pathology, University of Virginia, School of Medicine, Charlottesville, Virginia

Received for publication August 11, 1959

In 1955 Frenk, Antonowicz, Craig and Metcoff (1955) demonstrated that 6-dimethylamino 9(3′amino β D-ribofuranosyl) purine, designated as 6-aminonucleoside (AN) produces a nephrotic syndrome in young rats on subcutaneous injection. This aminonucleoside is related to puromycin, an experimental antibiotic from *Actinomyces alboniger* (Baker, Joseph and William, 1958). In AN-nephrotic rats the electrolyte composition of the body (Frenk *et al.*, 1955), the changes in protein and lipid composition of the serum and changes of certain enzyme systems in the kidneys have been studied (Fisher and Gruhn, 1958; Fisher, Tsuji and Gruber, 1958; Fiegelson, Drake and Recant 1957; Wilson, 1958). Quite recently Hartman, Hartman and Baldridge (1959), after investigating the effect of adenine on the nephrosis produced in rats by AN, suggested that the AN acts as an antimetabolite in some phases of nucleotide or nucleic acid synthesis.

The experiments presented here were designed to study the sequential relationship between the individual manifestations of the nephrotic syndrome as they develop in rats injected with AN. The problems investigated were: The appearance of functional or morphological alteration in the nephron prior to or concomitantly with changes in the composition of blood and urine; the relationship of the decrease of serum proteins to an increased urinary protein excretion or possibly to a change in protein metabolism and the relationship between the changes in serum lipid and protein concentration and the occurrence of stainable fat in the kidneys. Since a disturbance in the lipid metabolism of the liver in nephrotic rats has been demonstrated previously (Marsh and Drabkin, 1955), enzymatic and morphological changes in livers in AN-treated rats were also studied.

MATERIALS AND METHODS

In two sets of experiments 50 young adult Wistar rats of either sex, weighing 70–80 g. at the beginning of the experiment, were rendered nephrotic by daily subcutaneous injection of 0·3 ml./100 g. body weight of a 0·5 per cent solution of 6-aminonucleoside. An additional 20 rats served as controls. The rats were kept on a diet of Purina chow and water *ad lib.* One female and one male rat were killed 5 hr. after the first injection. Similar pairs were killed daily thereafter for the next 6 days and then at intervals of 2 or 3 days. The experiment was terminated on the 29th day. One day before being killed the rats were put into individual metabolic cages and 24 hr. specimens of urine were collected. The rats were killed by dislocating the neck, and blood was drawn immediately by cardiac puncture. After withdrawing ascitic fluid, when present, the body weight and the weights of livers and kidneys were taken. Two samples from these organs, and also from spleens, adrenals, lungs and hearts were fixed, one in alcohol and a second in formalin for histological and histochemical investigation. Other fresh samples of kidneys and of livers were immediately submitted to chemical

and histochemical estimation of succinic dehydrogenase. The dry weights of these tissues were determined.

Proteins and lipoproteins of serum and urine

The total protein in serum was estimated by a modified specific gravity gradient method of Linderström-Lang (Hoch and Marrack, 1945). The total protein in the urine was not measured. The 24 hr. urine was concentrated to 0·3–0·5 ml. by evaporation. The protein and lipoprotein distributions both in serum and urine were obtained by paper electrophoresis (Durrum, 1950) on Whatman 3 MM filter paper in veronal buffer of pH 8·6 and μ 0·05 (7 hr. at 7 volt/cm.). Bromphenol blue was used as the stain for protein and Oil Red O and/or Sudan black for lipoprotein. After recording the shape of the protein pattern with a Photovolt Densitometer, the paper was cut into strips at the positions of minimum staining, the dye was extracted with 0·01 N NaOH and the amount determined spectrophotometrically at 590 mμ. The lipoproteins were not determined chemically but their relative amounts were estimated by comparing the intensities of the staining and the areas underneath the peaks of the scanning diagrams.

Chemical and histochemical determination of enzyme activities

Succinic dehydrogenase in kidney and liver was determined in 20 μ thick frozen sections after incubation with 0·5 per cent sodium succinate and 0·1 per cent neotetrazolium chloride in phosphate buffered normal saline at pH 7·6. The specimens were gently agitated at 37° for 30 min. or for 1 hr. and then mounted for histological study. For chemical estimation, the reduced tetrazolium from 4–6 sections was extracted 3 times with acetone. The volume of the solution was adjusted to 10 ml. and the colour measured at 490 mμ. The concentration was expressed as μg. reduced tetrazolium per mg. of dried tissue. The 30 min. values in general were slightly more than half of the 1 hr. values, indicating a slight decrease of activity during the second 30 min.

Alkaline phosphatase was studied histochemically by the method of Gomori (1949) using sodium glycerophosphate as substrate at pH 9.

Chemical and histochemical investigation of lipids

The total lipids of 200–300 mg. wet weight of kidney or liver homogenate were extracted with a 3 : 1 alcohol-ether mixture at room temperature. After centrifugation the solvent was evaporated, the residue extracted with petroleum ether, in which the non-lipids are insoluble, and the extract evaporated and weighed (Kien and Wetzler-Ligeti, 1935).

The lipid in the frozen sections of formalin-fixed tissue was stained with Oil Red O and Sudan black.

Other procedures

Routine procedures were used for weights, dry-weights and histological examination. Periodic acid-Schiff (PAS) stain was used for mucopolysaccharides (McManus, 1946).

RESULTS

The succinic dehydrogenase activities in both the kidneys and in the livers of control rats were significantly higher in the males than in the females. A similar sex difference for the catalase content in the liver of mice has been observed previously (Adams, 1950). The response of male rats to injection of AN was less severe than that of females in all parameters studied. The occurrence of ascites was somewhat delayed, and a few males showed spontaneous recovery.

Massive proteinuria was found in all rats sacrificed after the 6th day. Visible ascites were present in the rats from the 10th day onward. One female rat had ascites on the 7th day. Two male rats, on the 20th and 29th day respectively, had resorbed their apparent ascites. Two male and 4 female rats died between the 20th and 29th days.

Findings suitable for statistical treatment are summarized in Table I for female and in Table II for male rats. The data for AN-treated rats before and after occurrence of ascites are analyzed separately. The significance of the findings was evaluated by the use of the simplified statistics for small numbers of observations (Dean and Dixon, 1951).

The observations with AN-treated rats were grouped into 3 consecutive periods :

Changes preceding the occurrence of increased proteinuria (1 *to* 6 *days*)

In rats slight proteinuria, which is more pronounced in the male, is physiological (Addis, MacKay and MacKay, 1926). It was present in the control and in treated rats in this early period (Fig. 1 and 2).

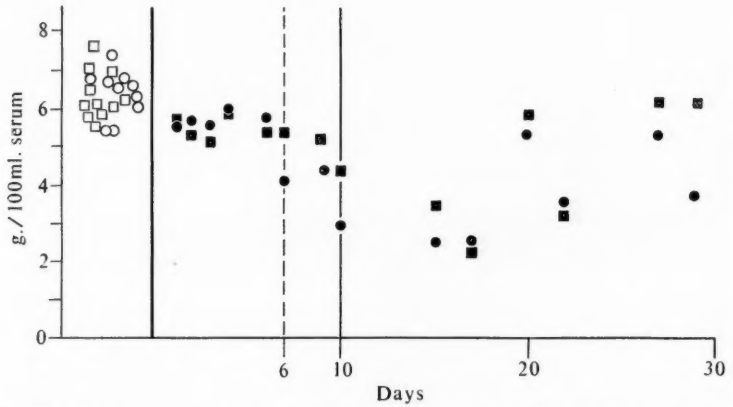


FIG. 3.—Total serum protein in control rats and in rats treated with 6-aminonucleoside.

□ = control males ○ = control females
■ = treated males ● = treated females

On the day after the first injection of AN the concentrations of the total serum proteins were decreased (Fig. 3), but no definite alterations in the concentrations of individual protein components were seen, and no increase in the serum lipoproteins could be demonstrated.

One day after injection of AN the succinic dehydrogenase activity of kidney slices was markedly reduced when determined by chemical analysis (Fig. 4 and 5). On histochemical investigation no difference in the intensity of the colour of reduced tetrazolium present could be observed.

In the proximal convoluted tubules an increase in size and in the number of PAS positive granules was observed (Fig. 10 and 11).

The weights of kidneys and livers as related to body weights did not change during this period.

Changes occurring concomitantly with massive proteinuria (6 *to* 10 *days*)

The decrease of serum protein concentration became progressively more severe, and an increase of α_2 and β globulin occurred. In many cases these two fractions

formed a single band (Fig. 6 and 7). On the 6th day stainable fat was observed in the kidney sections for the first time. Fat globules were localized in the lumina of glomerular capillaries and also in the lumina of arterioles but not in the renal epithelium. From the 8th day fat droplets were seen in the capillary endothelium of glomeruli. The glomerular basal membranes were thickened, and splitting of

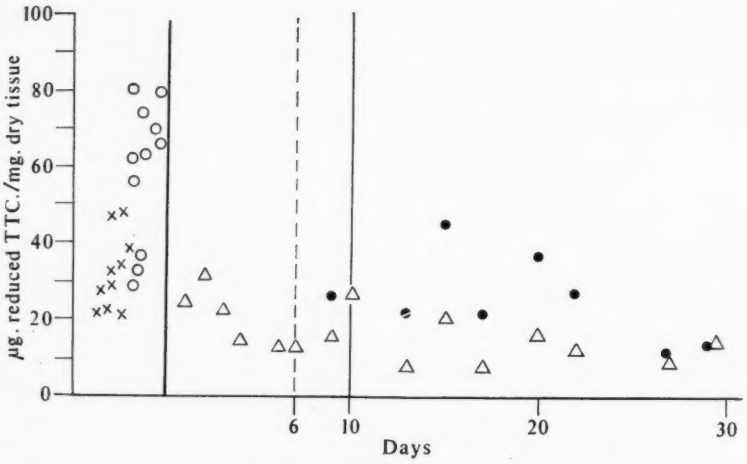


FIG. 4.—Succinic dehydrogenase activity of the kidneys of control and treated female rats.
Enzyme activity during ½ hr.: control = ×, treated = △
Enzyme activity during 1 hr.: control = ○, treated = ●
The division at the 6th day designates the occurrence of massive proteinuria, that at the 10th day the occurrence of ascites.

EXPLANATION OF PLATES.

FIG. 1.—Protein and lipoprotein distribution in serum and urine of control rats. The total protein is g./100 ml. serum; the other values are per cent of total protein.

FIG. 2.—Protein and lipoprotein distribution in serum and urine of control rats. The total protein is g./100 ml. serum; the other values are per cent of total protein.

FIG. 6.—Protein and lipoprotein distribution in serum and urine of rats treated with aminonucleoside. The total protein is g./100 ml. serum; the other values are per cent of total protein.

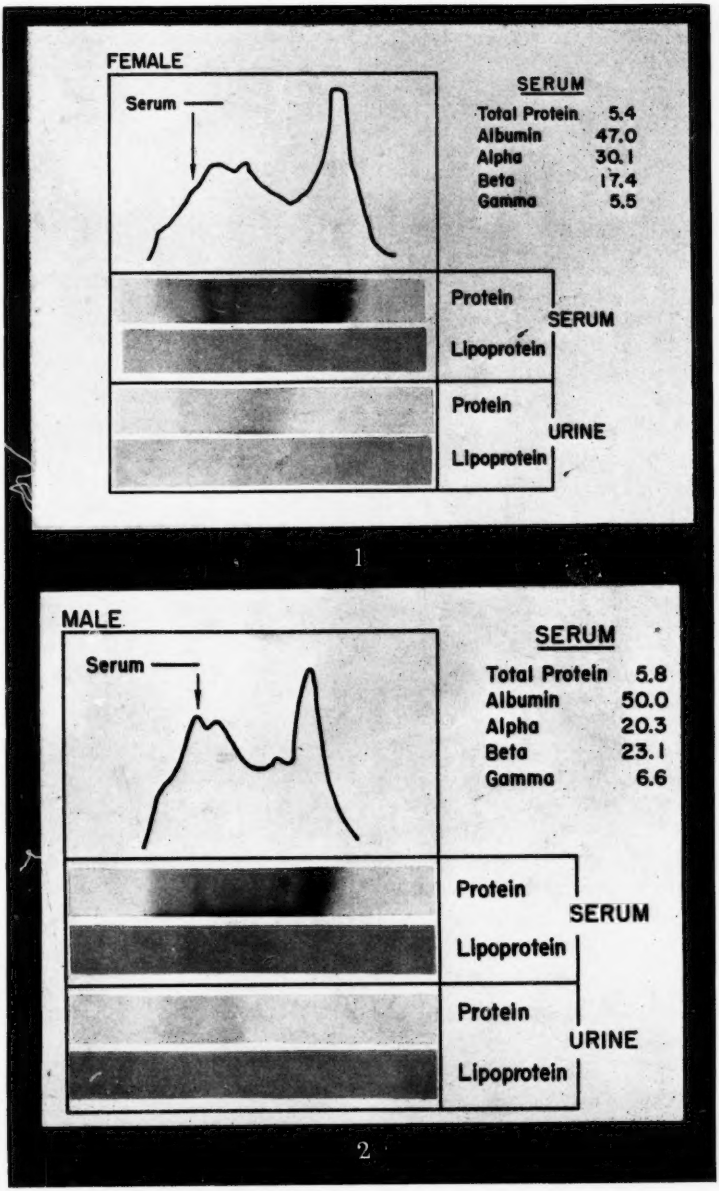
FIG. 7.—Protein and lipoprotein distribution in serum and urine of rats treated with aminonucleoside. The total protein is g./100 ml. serum; the other values are per cent of total protein.

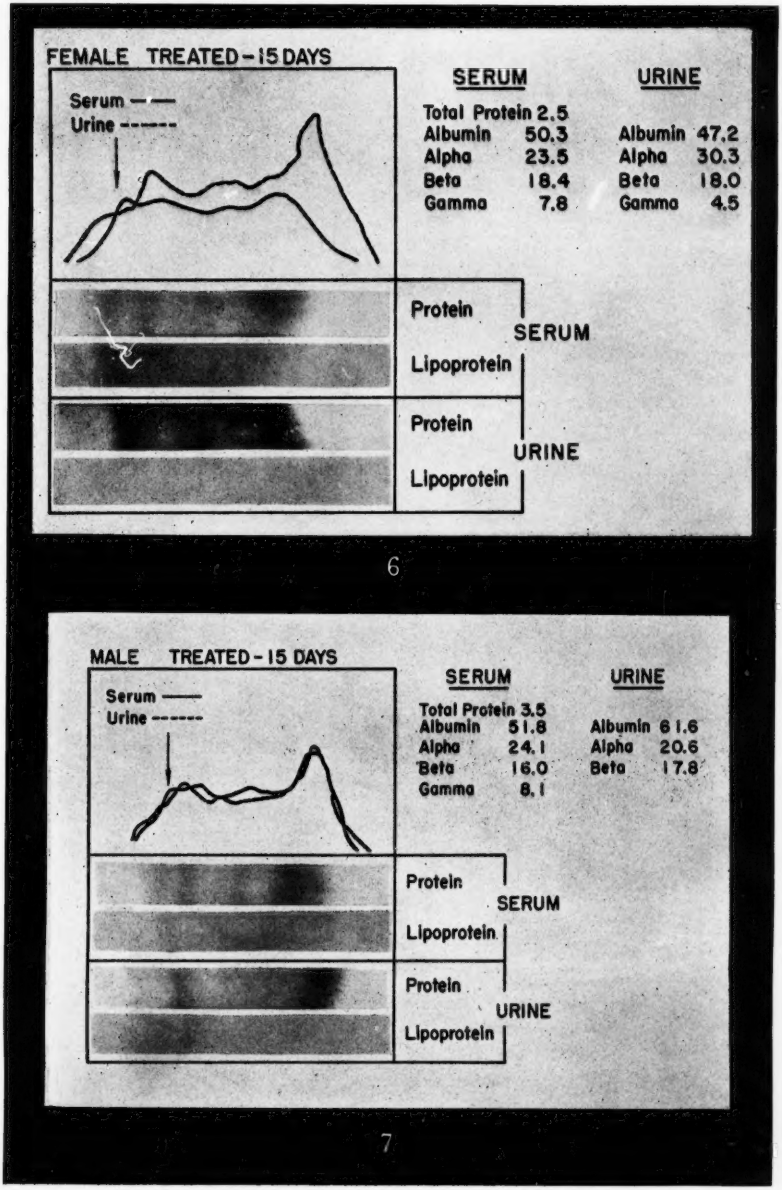
FIG. 10.—Kidney of control rat showing glomerulus and small number of granules in the proximal convoluted tubules. PAS stain, ×510.

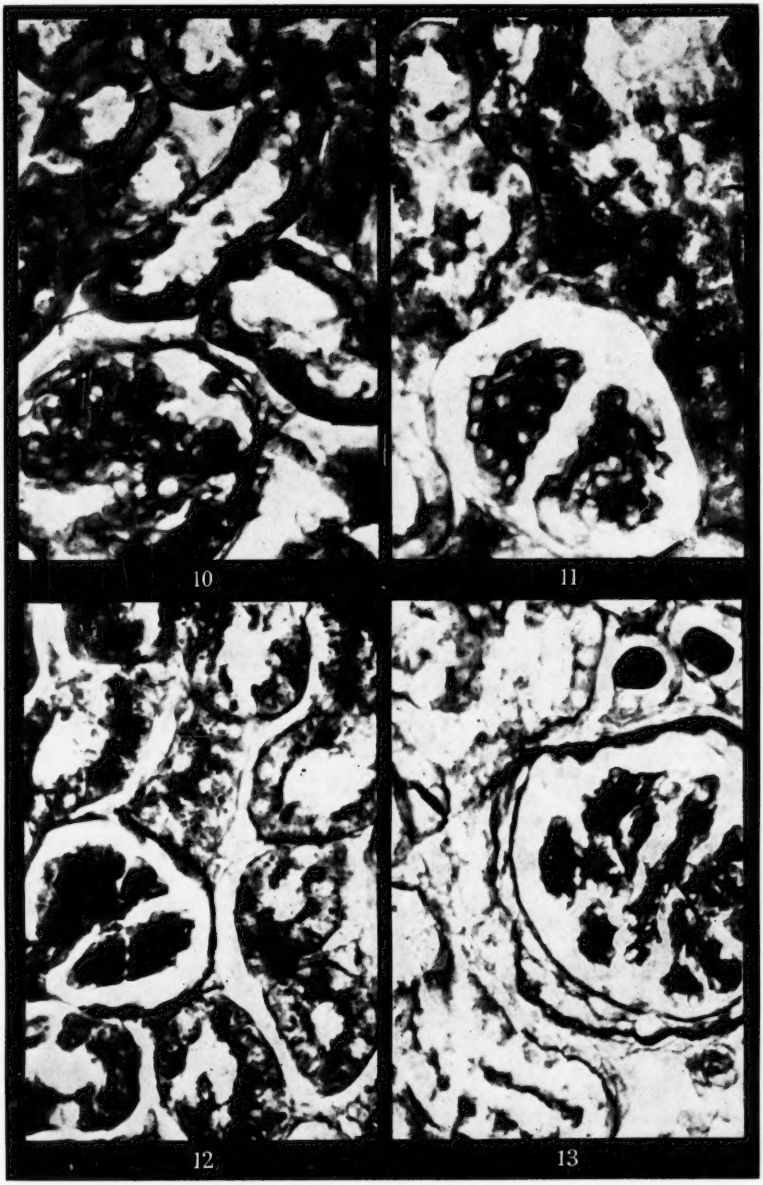
FIG. 11.—Kidney of rat treated 4 days with 6-aminonucleoside. The glomerulus does not show any changes; the number and size of granules in proximal convoluted tubules are increased. PAS stain, ×510.

FIG. 12.—Kidney of rat treated 7 days with 6-aminonucleoside. The glomerular basal membrane is thickened; the number of granules in proximal convoluted tubules is very markedly increased. PAS stain, ×510.

FIG. 13.—Kidney of rat treated 29 days with 6-aminonucleoside. The glomerular basal membrane is thickened; the basal membrane of Bowman's capsule shows reduplication. The number of granules in the proximal tubules is decreased. The tubular epithelium is flattened and the tubular lumina contain casts. PAS stain, ×510.


FEMALE
Serum
SERUM
Total Protein 5.4
Albumin 47.0
Alpha 30.1
Beta 17.4
Gamma 5.5
Protein
Lipoprotein
SERUM
Protein
Lipoprotein
URINE
1
MALE
Serum
SERUM
Total Protein 5.8
Albumin 50.0
Alpha 20.3
Beta 23.1
Gamma 6.6
Protein
Lipoprotein
SERUM
Protein
Lipoprotein
URINE
2


FEMALE TREATED - 15 DAYS
Serum
Urine
SERUM
Total Protein 2.5
Albumin 50.3
Alpha 23.5
Beta 18.4
Gamma 7.8
URINE
Albumin 47.2
Alpha 30.3
Beta 18.0
Gamma 4.5
Protein
SERUM
Lipoprotein
Protein
URINE
Lipoprotein
6
MALE TREATED - 15 DAYS
Serum
Urine
SERUM
Total Protein 3.5
Albumin 51.8
Alpha 24.1
Beta 16.0
Gamma 8.1
URINE
Albumin 61.6
Alpha 20.6
Beta 17.8
Protein
SERUM
Lipoprotein
Protein
URINE
Lipoprotein
7



10 11

12 13

Hoch-Ligeti.

TABLE I.—*Composition and Enzymatic Activity of Kidneys and Livers of Nephrotic Female Rats*

		Control (10 rats)		Treated: 1–9 days (8 rats)			Treated: 10–29 days (8 rats)		
		Range	Median*	Range	Median	*P*	Range	Median	*P*
Body weight (g.)		70–168	108±23·0	54–92	82±11·0	0·05	64–107	86·5±12·5	0·05
Kidney wt.	g/100 g. body	0·99–1·42	1·17±0·14	0·93–1·70	1·13±0·33	—	1·02–2·12	1·85±0·47	0·01
Liver wt.	g/100 g. body	4·30–5·45	4·93±0·26	4·40–6·75	5·25±0·68	—	3·33–9·06	6·62±1·66	0·05
Kidney dry wt.	mg./100 g. wet wt.	23·0–30·5	24·0±2·5	18·7–24·7	23·3±2·6	—	17·2–27·8	18·8±4·6	0·01
Liver dry wt.	mg./100 g. wet wt.	22·7–33·2	29·9±2·4	25·0–31·0	28·9±1·7	—	24·8–31·8	29·3±2·0	—
Kidney fat	mg./100 mg. wet wt.	1·19–6·50	4·69±1·8	2·66–6·10	4·0±2·9	—	1·13–4·0	2·89±1·2	0·01
Liver fat	mg./100 mg. wet wt.	3·27–6·36	4·48±0·71	2·62–4·93	3·75±0·76	0·05	3·46–5·17	4·24±0·50	—
Kidney fat	mg./100 mg. dry wt.	12·7–26·3	18·4±3·5	10·8–25·6	17·7±7·6	—	6·4–20·5	14·8±3·1	0·05
Liver fat	mg./100 mg. dry wt.	11·5–20·8	15·3±2·1	6·7–19·3	10·9±4·2	0·05	12·3–18·8	14·6±1·9	—
Kidney TTC (½†)	μg./mg. dry wt.	21·4–48·5	31·2±9·0	13·2–31·6	16·4±9·4	0·01	8·5–27·5	14·2±8·2	0·01
Kidney TTC (1‡)	μg./mg. dry wt.	29·0–80·5	61·3±17·0	—	—	—	11·9–47·0	25·3±15·1	0·01
Liver TTC (½)	μg./mg. dry wt.	17·3–39·5	24·8±5·8	13·5–26·0	18·3±4·1	0·05	21·8–40·0	28·6±6·0	—
Liver TTC (1)	μg./mg. dry wt.	28·8–67·0	53·6±11·1	—	—	—	37·5–70·2	47·0±10·8	—
Total protein (g./100 ml. serum)		5·4–7·4	6·6±0·66	4·1–6·0	5·6±0·97	0·01	2·5–5·4	3·6±1·48	0·01

* The confidence limits are for $P = 0·05$, except where a difference is significant on the 1 per cent level, in which case they are given for $P = 0·01$.

† TTC (½): Enzymatic reduction of tetrazolium chloride in 30 min.

‡ TTC (1): Enzymatic reduction of tetrazolium chloride in 1 hr.

TABLE II.—*Composition and Enzymatic Activity of Kidneys and Livers of Nephrotic Male Rats*

		Control (10 rats)		Treated: 1–9 days (8 rats)			Treated: 10–29 days (8 rats)		
		Range	Median*	Range	Median	*P*	Range	Median	*P*
Body weight (g.)		88–172	106·5±19·3	55–115	95±17	—	105–135	116±8·7	—
Kidney wt.	g./100 g. body	0·96–1·47	1·11±0·12	0·98–1·48	1·21±0·15	—	1·06–2·06	1·44±0·31	0·05
Liver wt.	g./100 g. body	4·3–6·7	4·99±0·57	4·0–6·8	5·53±0·81	—	3·5–8·7	6·45±1·5	—
Kidney dry wt.	mg./100 g. wet wt.	21·7–24·8	23·9±0·71	13·6–25·3	21·8±3·4	—	16·8–24·4	21·7±1·9	0·05
Liver dry wt.	mg./100 g. wet wt.	23·7–33·0	29·7±2·1	21·9–29·1	27·8±2·1	—	21·0–30·4	29·2±2·3	—
Kidney fat	mg./100 mg. wet wt.	2·71–11·0	3·85±1·9	3·44–6·25	4·32±1·2	—	2·58–5·8	3·30±0·93	—
Liver fat	mg./100 mg. wet wt.	2·85–4·90	3·62±0·47	2·57–5·35	3·64±0·81	—	2·59–4·78	3·24±0·64	—
Kidney fat	mg./100 mg. dry wt.	11·3–31·7	15·8±4·7	15·7–27·0	20·6±4·5	0·05	13·8–26·9	17·4±3·8	—
Liver fat	mg./100 mg. dry wt.	10·3–15·5	12·4±1·26	9·1–24·9	13·7±5·2	—	9·7–16·2	12·3±1·9	—
Kidney TTC (½)†	μg./mg. dry wt.	26·0–47·5	36·1±7·1	11·3–26·9	18·7±6·7	0·01	12·4–41·0	18·3±12·3	0·01
Kidney TTC (1)‡	μg./mg. dry wt.	32·0–104·0	77·8±23·8	—	—	—	26·6–63·0	32·7±15·6	0·01
Liver TTC (½)	μg./mg. dry wt.	12·5–39·6	26·7±6·3	9·5–26·8	14·4±5·0	0·05	19·2–32·5	29·0±4·2	—
Liver TTC (1)	μg./mg. dry wt.	23·6–76·5	63·2±122	—	—	—	38·0–74·5	52·8±10·6	—
Total protein (g./100 ml. serum)		5·5–7·4	6·1±0·63	5·1–6·0	5·4±0·46	0·01	2·3–6·3	4·0±1·6	0·01

* The confidence limits are for $P = 0{\cdot}05$, except where a difference is significant on the 1 per cent level, in which case they are given for $P = 0{\cdot}01$.

† TTC (½): Enzymatic reduction of tetrazolium chloride in 30 min.

‡ TTC (1): Enzymatic reduction of tetrazolium chloride in 1 hr.

the glomerular basal membrane could be demonstrated with PAS stain in many instances.

Stainable fat was found in the liver at the same time as in the kidneys. The fat occurred in the lumina of the central and interlobular veins. From the 8th day on, the Kupffer cells contained phagocytosed droplets.

On chemical estimation the fat content of the kidneys was not significantly changed. The fat content of the liver of female rats was slightly decreased during this period.

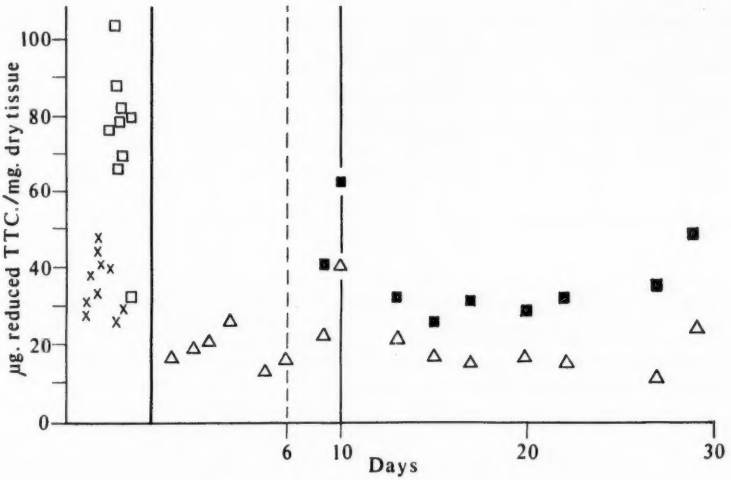


FIG. 5.—Succinic dehydrogenase activity of the kidneys of control and treated male rats.
Enzyme activity during ½ hr.: control = ×, treated = △
Enzyme activity during 1 hr.: control = □, treated = ■
The division at the 6th day designates the occurrence of massive proteinuria, that at the 10th day the occurrence of ascites.

All serum protein components could be demonstrated in the urine by electrophoretic analysis, with preponderance of albumin. None, or only traces of stainable lipoproteins were present.

The histological and histochemical alterations of the kidneys described above became progressively more prominent (Fig. 12).

Changes observed after occurrence of ascites (after the 10th day)

The weight and the water content of the kidneys of all rats and the wet weights of the livers of female rats increased (Fig. 8 and 9).

Internal hydronephrosis with flattening of the tubular epithelium and dilatation of the tubules was present, and the tubular lumina were filled with PAS positive protein casts.

Fat droplets were now also present in the tubular epithelium, occurring mostly in the proximal convoluted tubules. The droplets were localized subnuclearly. The PAS positive granules in the proximal convoluted tubules which were so

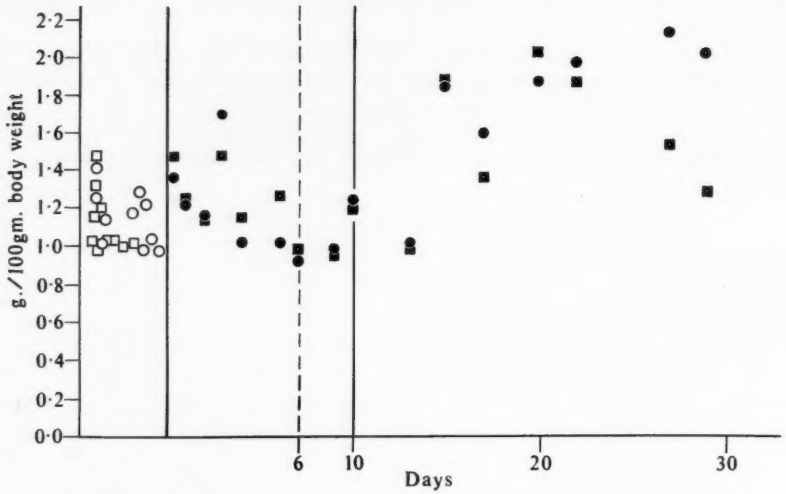


FIG. 8.—Kidney weights of control rats and of rats treated with 6-aminonucleoside.
□ = control males ○ = control females
■ = treated males ● = treated females
The division at the 6th day designates the occurrence of massive proteinuria, that at the 10th day the occurrence of ascites.

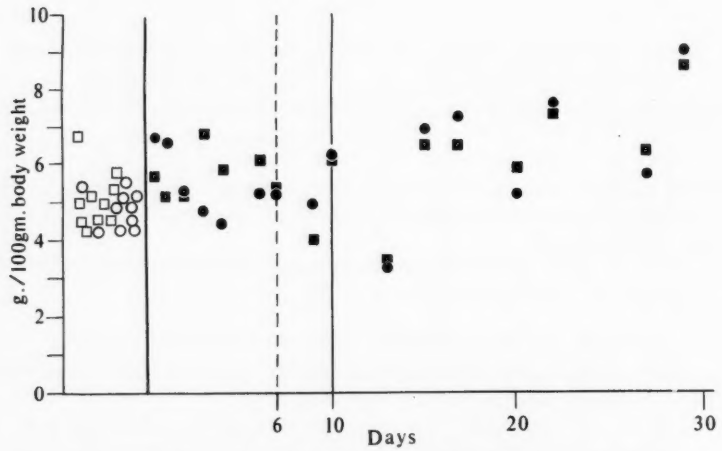


FIG. 9.—Liver weights of control rats and of rats treated with 6-aminonucleoside.
□ = control males ○ = control females
■ = treated males ● = treated females
The division at the 6th day designates the occurrence of massive proteinuria, that at the 10th day the occurrence of ascites.

prominent in the earlier phases of the experiment became scanty. The glomerular basal membrane changes were progressive, and most glomeruli showed splitting of the parietal basal membrane (Fig. 13).

The alterations in the enzymatic activity and in the proteins of the serum and urine described were progressive.

The fat content of kidneys by chemical analysis was slightly decreased in female rats. The fat contents in the kidneys of male rats and in the livers of all rats were unchanged.

During the entire course of the experiment the succinic dehydrogenase contents of the livers of male rats did not show a significant alteration. In the female rats the liver succinic dehydrogenase activity was slightly decreased in the early phase of the experiment. The body weights of female rats were lower than those of the controls ; the body weights of male rats did not differ from the controls.

No changes in the alkaline phosphatase activities of kidney or liver cells could be demonstrated by histochemical methods.

DISCUSSION

The decrease in the total serum protein concentration occurring 24 hr. after injection of AN could be accounted for either by an excretion of protein, by an increase of the water content of the serum or by a change in the protein metabolism. Changes in protein metabolism were demonstrated in rats rendered nephrotic by injection of anti-rat kidney rabbit serum (Drabkin and Marsh, 1955). It is possible that in the present experiment an increase in the water content of the serum precedes the increase of the water content of the parenchymatous organs which could be demonstrated only at the later part of the experiment. The increase of PAS positive granules in the proximal convoluted tubules pointed to an increased tubular reabsorption of proteins (Oliver, MacDowell and Lee, 1954), which in turn suggested that more than the usual amount of protein may have passed through the glomerular filter. Even after massive proteinuria occurred on the 6th day of the experiment, an increased reabsorptive activity of the tubular epithelium was indicated by a still more pronounced increase in size and number of PAS positive granules in the epithelium of the tubules. Only after about the 20th day of the experiment did the number of these granules decrease in the epithelium, suggesting an exhaustion phenomenon.

The suppression of succinic dehydrogenase activity in the kidneys seemed to be due to the direct toxicity of AN on the tubular epithelium, although a slight effect was observed on the liver cells of the female rats. The significance of the decrease in the succinic dehydrogenase activity in the development of the nephrotic syndrome cannot be evaluated at this time.

The alkaline phosphatase activity of tubular epithelium is thought to be connected with the tubular reabsorption of carbohydrates (Wilmer, 1944). The absence of any effect of the injection of AN on the alkaline phosphatase is in agreement with a lack of influence of AN on the excretion of carbohydrates.

The mechanism underlying the occurrence of hyperlipemia and hypercholesteremia in rats rendered nephrotic by administration of anti-kidney serum was investigated by Rosenman, Friedman and Byers (1956), and parallelism between the severity of albuminuria and hyperlipemia was found. The observation (Rosenman, Friedman and Byers, 1955), that in nephrotic rats the increase in the lipids

is restricted to the serum has been confirmed by the present investigation. Fat droplets could be demonstrated tinctorially in the kidneys of nephrotic rats from the 6th day onward, first in the blood stream, then in the capillary endothelium, and finally in the tubular epithelium. The amount of lipid in the serum, or in the individual cells of the tubules, however, is too small to contribute more than a fraction of a per cent in the chemical estimates of the total lipids in the organ.

As has been observed in human nephrotic patients, the increase of lipoproteins in the serum is not accompanied by an increase of urinary lipoprotein excretion (Hardwicke, 1954; Lewis, 1955). It has been suggested previously (Lewis, 1955; Oncley, Gurd and Melin, 1950; Eder, 1957) that the low density β-lipoproteins do not pass the glomerular filter because of their large molecular size (over 2,000,000). The lipoproteins in the serum of nephrotic rats appeared in the electrophoretic patterns as a wide band in the position of globulins without definite peaks in any of their fractions. They also contained, in all likelihood, high density lipoproteins of lower molecular weight. Since in the electrophoretic pattern of the urinary proteins all fractions of serum globulins could be demonstrated, thus showing an increased glomerular permeability for larger molecules, it is suggested that the lipoproteins are either selectively retained or that these molecules may be split, presumably in the kidneys, so that only the globulin component is excreted.

SUMMARY

In rats rendered nephrotic by subcutaneous injection of 6-aminonucleoside the sequential relationship of alterations in function and morphology of kidneys and livers to the protein and lipid content in serum and urine were investigated.

It was found that a decrease in the total serum proteins and a decrease in the succinic dehydrogenase activity, together with signs of an increase of protein reabsorption in the kidneys, precedes the proteinuria.

An increase of lipoproteins in serum, the presence of stainable fat in the kidneys and in the livers, and an alteration of the glomerular basal membrane occurred concomitantly with massive proteinuria.

An increase in the water content of the kidneys, reduplication of glomerular basal membranes, internal hydronephrosis and signs of decreased tubular reabsorption were observed after the occurrence of ascites.

The fat content of the kidneys by chemical estimation was found to be decreased in female rats.

In control male rats the succinic dehydrogenase activity of kidneys and livers was significantly higher than that of female rats.

REFERENCES

Adams, D. H.—(1950) *Brit. J. Cancer*, **4**, 183.
Addis, T., MacKay, E. M. and MacKay, L. L.—(1926) *J. biol. Chem.*, **71**, 139.
Baker, B. R., Joseph, J. P. and William, J. H.—(1958) *J. Amer. chem. Soc.*, **76**, 2838.
Dean, R. B. and Dixon, W. J.—(1951) *Anal. Chem.*, **23**, 636.
Drabkin, D. and Marsh, J.—(1955) *J. biol. Chem.*, **212**, 623.
Durrum, E. L.—(1950) *J. Amer. chem. Soc.*, **72**, 2943.
Eder, H. A.—(1957) *Amer. J. Med.*, **23**, 269.

FIEGELSON, E. B., DRAKE, J. W. AND RECANT, L.—(1957) *J. Lab. clin. Med.*, **50**, 437.
FISHER, E. R. AND GRUHN, J.—(1958) *Arch. Path.* (*Lab. Med.*), **65**, 545.
Idem, TSUJI, F. I. AND GRUBER, J.—(1958) *Proc. Soc. exp. Biol., N.Y.*, **97**, 448.
FRENK, S., ANTONOWICZ, I., CRAIG, J. M. AND METCOFF, J.—(1955) *Ibid.*, **89**, 424.
GOMORI, G.—(1949) *Ibid.*, **70**, 7.
HARDWICKE, J.—(1954) *Proc. R. Soc. Med.*, **47**, 832.
HARTMAN, M. E., HARTMAN, J. D. AND BALDRIDGE, R. C.—(1959) *Proc. Soc. exp. Biol., N.Y.*, **100**, 152.
HOCH, H. AND MARRACK, J. R.—(1945) *Brit. med. J.*, ii, 876.
KIEN, G. AND WETZLER-LIGETI, C.—(1935) *Wien. klin. Wschr.*, **48**, 871.
LEWIS, A. L.—(1955) *Med. Clin. N. Amer.*, **39**, 1015.
MARSH, J. AND DRABKIN, D.—(1955) *J. biol. Chem.*, **212**, 633.
MCMANUS, J. A.—(1946) *Nature, Lond.*, **158**, 202.
OLIVER, J., MACDOWELL, M. AND LEE, Y. CH.—(1954) *J. exp. Med.*, **99**, 589.
ONCLEY, J. L., GURD, F. R. N. AND MELIN, M.—(1950) *J. Amer. chem. Soc.*, **72**, 458.
ROSENMAN, R. H., FRIEDMAN, M. AND BYERS, S. O.—(1955) *J. clin. Invest.*, **34**, 700.—(1956) *Ibid.*, **35**, 522.
WILMER, H. A.—(1944) *Arch. Path.* (*Lab. Med.*), **37**, 227.
WILSON, S. G. F.—(1958) *Pediatrics*, **21**, 963.

SOME APPLICATIONS OF A SINGLE-DILUTION METHOD OF TITRATING NEUROTROPIC VIRUSES IN ZERO MORTALITY (D_0) UNITS

D. J. BAUER

From the Wellcome Laboratories of Tropical Medicine, Euston Road, London, N.W.1

Received for publication August 27, 1959

THE 50 per cent end-point is an article of faith in virology. Practically every quantitative determination, whether of virus titre, estimation of content of neutralizing antibody, or investigation of antiviral chemotherapeutic effect, is based upon an experimental design which involves the determination of a 50 per cent end-point. In view of the inaccuracy, artificial nature and practical disadvantages of the method it is of interest to enquire how its use has become so widespread.

The concept of the LD50 was first introduced by Trevan (1927) in order to improve upon the imprecision of the minimal lethal dose, which had become firmly established in pharmacological investigation as the method of determining toxicity. The occurrence or non-occurrence of death is a quantal response, and each test animal can yield only 2 pieces of information. Any quantitative method which rests upon such a limited basis therefore suffers from inherent lack of precision which cannot be remedied, and this applies not only to the minimal lethal dose but also to the more refined concept of the LD50. The factor which has done more than anything else to establish the 50 per cent end-point as the method of choice in virology was the work of Reed and Muench (1938), which, although it consisted of a hypothetical illustration from serology, has become the most widely quoted source in virological literature. Shortly afterwards Gard (1940) showed that the reciprocal of the incubation period in mice infected with the GD VII and FA strains of mouse encephalomyelitis virus had a linear regression on the logarithm of the virus dilution, and this relation could be used as a means of titrating virus suspensions of unknown infectivity by interpolation from a previously determined response curve. In spite of the many advantages of this method it has not come into general use, and the purpose of the present work is to show that the procedure proposed by Gard is applicable to the titration of many other neurotropic viruses and, in a more refined form which involves measurements in units of zero mortality, possesses many advantages over the determination of the LD50.

MATERIALS AND METHODS

Viruses.—The origin of the viruses used in the present work has been described in an earlier communication (Bauer and Bradley, 1956).

Mice.—The work was carried out with commercial Swiss white mice of varying weights obtained from a number of different dealers.

Dose-response curves.—Serial decimal dilutions of the virus preparation, which usually consisted of infected mouse brain, were inoculated intracerebrally into groups of 6–12 mice, beginning either at the highest dilution, or at the lowest dilution using a separate syringe

for each dilution. The animals were observed for up to 3 weeks, and the deaths which occurred were recorded at 9 a.m. and 5 p.m. each day ; a mouse found dead in the morning was counted as having lived for an integral number of days, and when found dead in the afternoon for an extra half-day. Mice discarded alive and well at the end of the observation period were considered to have survived for infinite time for the purpose of calculating the mean response.

Yellow fever vaccine.—Five-dose ampoules of " Wellcome " brand yellow fever vaccine were selected at random from commercial stocks. The vaccine consists of a freeze-dried suspension of chick embryos infected with the 17D strain of yellow fever virus.

Yellow fever immune serum.—The immune serum was prepared by the hyperimmunization of rabbits and was presented by Dr. J. S. Porterfield of the National Institute for Medical Research, London.

RESULTS

Dose-response curve of neurovaccinia virus

It has previously been shown (Bauer, 1958) that in mice infected intracerebrally with the IHD strain of neurovaccinia virus there is a linear relationship between the reciprocal of the survival time and the logarithm of the dilution of virus inoculated, and that the regularity of the relationship was such that it was

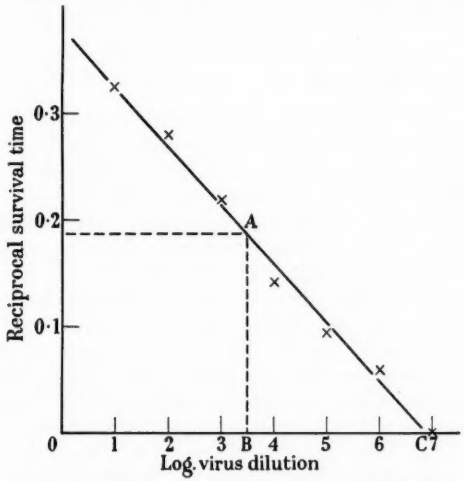


FIG. 1.—Dose-response curve of neurovaccinia virus in mice. The points represent the mean reciprocal survival times of groups of 6 mice.

possible to use it as the basis of a test of antiviral chemotherapeutic activity. In subsequent work the properties of the dose-response curve have been studied in greater detail. A typical example is given in Fig. 1. It should be noted that the curve does not approach the axis of abscissae asymptotically but gives an unambiguous intercept. At this point the ordinate is zero, since all the mice survive indefinitely, and the point therefore represents the LD 0. The LD 0 obtained by this method is a natural parameter of the dose-response curve, and thus differs radically from the LD 0 of the sigmoid mortality curve, which is indeterminate owing to the asymptotic approach, and in order to avoid confusion it is suggested that the point should be called the D_0, the dose of virus

which produces zero mortality. The LD50 is now indeterminate, since an indefinite number of ordinates can be consistent with 50 per cent survival once the survival time is incorporated into the response. The titre in D_0 units of the virus preparation used for obtaining the dose-response curve can be calculated from the regression coefficient (b) and the ordinate intercept (c) obtained by the least squares method, and is then $-b^{-1}c$. The titre of a virus preparation of unknown concentration can be calculated with the aid of the previously determined dose-response curve by a method given below provided that the slope of the curve, *i.e.* the regression coefficient, remains constant.

Consistency of the slope of the dose-response curve

The results of a number of determinations of the regression coefficients of the dose-response curves of neurovaccinia and other neurotropic viruses, carried out at intervals over a period of 6 yr., are given in the Table. The determinations were carried out with mice of several different strains, some of unknown origin, and no attempts were made to standardize the conditions, but it will be seen that the value of b for neurovaccinia is reasonably constant, with a standard error of 14 per cent.

Relationship of the D_0 to the LD50

The data used for constructing the dose-response curve can be used for calculating the LD50 provided that the 50 per cent mortality end-point is covered, and the relationship between the D_0 and the LD50 can thus be determined. In the titration of Fig. 1 the D_0 end-point was 6·92 log units and the LD50 end-point was 5·67 log units by the moving average method (Thompson, 1947) and approximately 5·75 log units by the Reed and Muench method, giving a mean of 5·71 log units. The LD50 therefore exceeded the D_0 by 1·21 log unit. In further experiments a standard preparation of neurovaccinia virus was titrated on 3 separate occasions, 191 mice being used in all, and the D_0 and LD50 titres were determined. Values of 7·9, 7·9 and 7·8 log units were obtained for the D_0 titre and 6·0, 6·3 and 6·0 for the LD50 titre. The reproducibility of the D_0 titration is therefore within acceptable limits, and the results in combination with the preceding experiment give a mean difference between the D_0 and the LD50 of 1·65 log unit. A titration of another standard preparation gave a difference of 1·60 log unit. The LD50 of neurovaccinia virus is therefore equivalent to approximately 40 D_0, and the titre in D_0 units can be converted to the familiar units if required, by subtracting 1·6 log unit.

Dose-response curves of other viruses

It has already been shown (Bauer, 1958) that a linear dose-response curve can be obtained with neurotropic ectromelia virus (pseudolymphocytic choriomeningitis), and further examples for other neurotropic viruses are given in the Table. In the present work it has been found that linear dose-response curves can also be obtained with herpes, the 17D strain of yellow fever, the Flury strain of rabies virus, Anopheles A and dengue I (Hawaiian strain) viruses. The appropriate numerical data are given in the Table, and the dose-response curves of the 17D and Flury strains, which as will be shown later, are of practical value

TABLE.—*Regression Coefficients of Dose-Response Curves of Neurotropic Viruses*

Virus	Number of determinations	Number of mice	Mean or individual value of regression coefficient (b)	Standard error of b
Neurovaccinia	28	1012	−0·043	0·006
Ectromelia	8	245	−0·033	0·003
Yellow fever (17D)	5	249	−0·016	0·005
Herpes simplex	7	202	−0·057	0·003
Rabies (Flury)	2	84	−0·031 }* −0·028 }	—
Ilhéus	1	61	−0·036*	—
Dengue I	1	36	−0·017*	—
Anopheles A	1	83	−0·016*	—

* Individual values.

in the titration of rabies and yellow fever vaccines, are illustrated separately in Fig. 2 and 3.

Calculation of titre in D_0 units

Consider the death of a single animal occurring at a time t after infection; t^{-1} then defines a point on the dose-response curve of Fig. 1 corresponding to the abscissa OB. The distance of B from C, the D_0 point, is the content of virus in

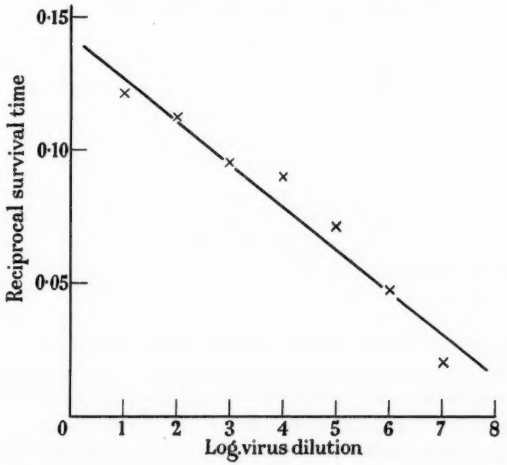


FIG. 2.—Dose-response curve of the 17D strain of yellow fever virus.

D_0 units, and is equal to $t^{-1}b^{-1}$. The survival time of a single animal is thus an experimental estimate of the titre, and the survival time of each animal in a group infected with the same dose provides its own independent estimate of the titre. The mean titre can thus be obtained from the mean reciprocal survival time of the group, and data are available for calculating the standard deviation. Moreover, the titre can be determined by the inoculation of a single dilution without previous knowledge of an expected end-point, and the failure often

encountered in LD50 titrations as a result of the use of an inappropriate range of dilutions cannot occur. A numerical example of the calculation of a titre by this method is given below.

Some applications of the D_0 titration

Single dilution titrations.—The method of obtaining the titre from a single dilution is particularly suitable for carrying out large numbers of independent titrations, such as determinations of the potency of batches of live virus vaccines. As an example, an ampoule of yellow fever vaccine was reconstituted to give a 10^{-1} dilution of the original material, diluted 1 : 100 to give a final dilution of

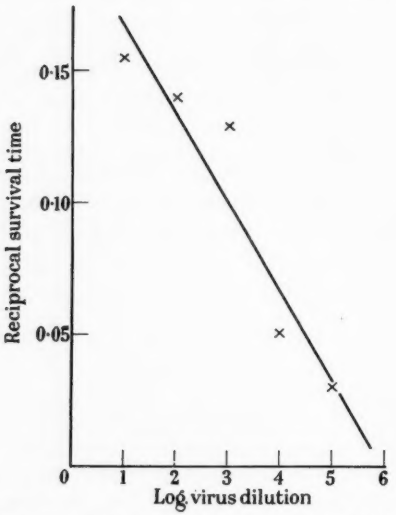


FIG. 3.—Dose-response curve of the Flury strain of rabies virus.

10^{-3}, and injected intracerebrally into 5 mice, which died after 8, 9, 11, 11 and 12 days. The mean reciprocal survival time was then 0·0835 which divided by the value of *b* for the 17D strain of yellow fever virus given in the Table (0·016) gave 5·22 as the logarithm of the titre in D_0 units.

Multiple dilution titrations.—The precision of the estimate of the titre can be increased by inoculating 2 or more dilutions, and the fate of each single animal contributes information, whereas in LD50 titrations only the dilutions enclosing the end-point can be used for calculation, the remaining animals being wasted. The value of *b* for the responses obtained from the titration or previously determined value of *b* can be used to calculate the D_0.

Determination of antiviral chemotherapeutic effect.—It has already been shown (Bauer, 1958) that treatment of infected animals with an agent possessing antiviral chemotherapeutic activity will displace the regression line to lower response levels. This results in an increase in the value of the D_0, and the extent of the increase in log units can be used as a measure of antiviral chemotherapeutic

activity. The method is then exactly comparable to a conventional 2 n-point pharmacological assay, where n is the number of dilutions used for inoculation.

Determinations of virus growth curves.—A commonly occurring requirement in research is the determination of virus titre in an infected organ at successive intervals of time after infection; if the LD50 end-point is used the procedure is very cumbersome, requires a large number of mice, and does not give very accurate results. The D_0 method affords a much simpler method of obtaining the required information by the process of transformation of regression coefficients. A number of mice are infected intracerebrally with a constant dose of virus; on successive days after infection 1 or more mice are killed, and the brains removed, pooled and prepared as a 10 per cent suspension. Each suspension thus prepared is inoculated intracerebrally into a group of mice, the size of the group depending upon the degree of accuracy required, and the survival times are recorded and converted into reciprocals. The coefficient of regression (b_1) of the reciprocal survival times on time in days after infection is then determined by the method of least squares and divided by b, the regression coefficient of the virus dose-response curve, giving b_2, the regression coefficient of the virus titre growth curve. This follows from the fact that the reciprocal survival times (t^{-1}) are converted into titres in D_0 units when divided by b; since the t^{-1} occur in both terms of the numerator of the expression for b_1, replacement of each response by $t^{-1}b^{-1}$ gives b_1b^{-1}, whence $b_2 = b_1b^{-1}$, where b_2 is the regression coefficient of the virus growth curve. The actual growth curve is obtained by adding 1 log unit to each transformed ordinate to allow for the initial 10^{-1} dilution.

Since the D_0 bears a constant relationship to the LD50 the growth curve in LD50 units, if required, can be obtained by subtracting a previously determined constant from each ordinate. An example of the method is shown in Fig. 4, in which the growth curve for neurovaccinia virus is given together with the reciprocal survival time regression line from which it was derived. The mean titres for each day were obtained by dividing the corresponding mean reciprocal survival times by b. The results afford the necessary data for determining the standard error of the regression coefficient and carrying out tests of linearity.

Investigation of the kinetics of virus inactivation.—In studies of the preservative effect of various diluents, or of the inactivating effect of physical and chemical agents, it is necessary to carry out serial determinations of titre at frequent intervals in order to obtain an inactivation curve. This can be done most easily by injecting the undiluted virus preparation into a group of animals as often as is necessary and determining the D_0 by the method given above. If inactivation is proceeding by a first-order reaction the velocity coefficient can be determined directly by the method of regression coefficient transformation. The occurrence of inactivation can be inferred from a calculation of the probability of the deviation of b from zero, and the significance of the difference in velocity coefficients obtained under different experimental conditions can be determined in a similar manner. The responses obtained are also suitable for carrying out an analysis of variance and test of linearity. An illustration of the method is given in Fig. 5, which illustrates the inactivation curve obtained with an ampoule of commercial yellow fever vaccine reconstituted with saline in accordance with the instructions for use and left at room temperature (16°). The material was inoculated into groups of 11–13 mice 5, 10, 20 and 40 min. after reconstitution, 47 mice being

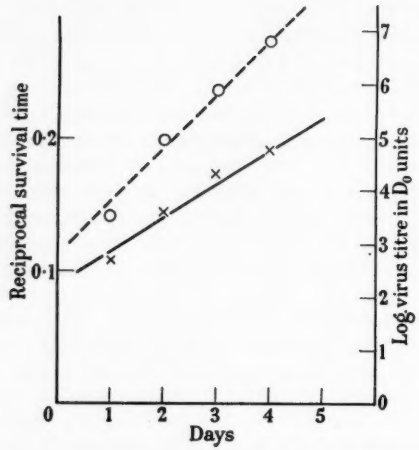


FIG. 4.—Growth curve of neurovaccinia virus in mouse brain. Upper line; regression of responses on time (see text). Lower line; growth curve of virus titre obtained by transformation of the response line. The points represent the mean responses and the mean titres obtained by transformation. *Ordinates*; left-hand, reciprocal survival time; right-hand, virus titre in D_0 units. *Abscissa*; time in days after infection.

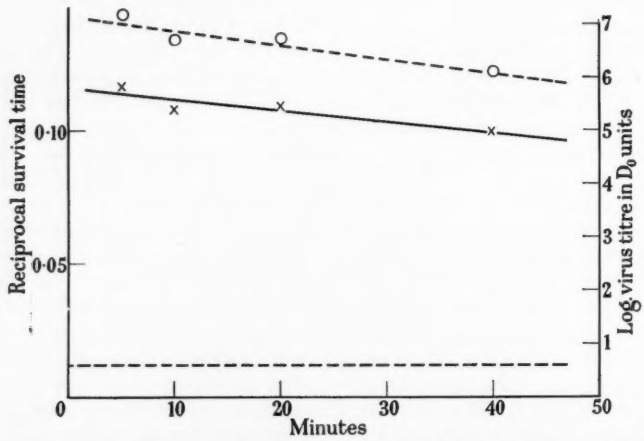


FIG. 5.—Thermal inactivation of yellow fever vaccine. Lower line; regression of responses on time. Upper line; regression of virus titre on time obtained by transformation. *Ordinates*; left-hand, reciprocal survival time; right-hand, virus titre in D_0 units. *Abscissa*; time of exposure in minutes. Dotted line; minimum permissible virus titre.

used in all. b_1 was found to be $-4{\cdot}409 \times 10^{-4}$, and the regression of the response on time is shown in the lower curve of Fig. 5. The variance of b_1 was $1{\cdot}532 \times 10^{-8}$ and the probability of the difference of b_1 from zero was then $< 0{\cdot}001$, 43 degrees of freedom being available for determining this quantity. The high level of significance shows that inactivation of the virus was taking place. The regression of virus titre in D_0 units on time (b_2) was then obtained by dividing b_1 and the means of the responses by $-0{\cdot}016$, the mean value of b for the 17D strain of yellow fever virus given in the Table, and is illustrated in the upper curve of Fig. 5. It can be seen that inactivation of virus to the extent of 0·8 log unit (in both D_0 and LD50 units) took place over 30 min., the recommended period of use for the reconstituted vaccine. The minimum permissible titre as required by inter-

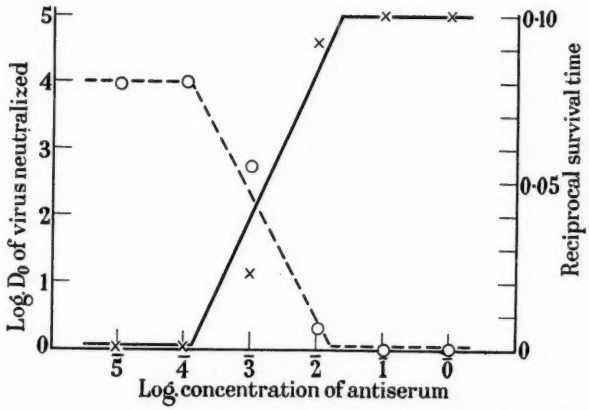


FIG. 6.—Neutralization curve of the 17D strain of yellow fever virus. *Ordinates*; left-hand, logarithm of amount of virus neutralized in D_0 units; right-hand, reciprocal survival time. *Abscissa*; logarithm of concentration of antibody in arbitrary units.

○ Mean Responses.
× Mean amounts of virus neutralized, obtained by transformation of the responses.

national regulations is given by the dotted line of Fig. 5, from which it can be seen that there is still a considerable excess of virus present in spite of the inactivation which has taken place.

The rate of loss of infectivity of chemically inactivated vaccines could also be determined by the same method.

Neutralization tests.—Virus neutralization tests are carried out by determining the LD50 end-point of a virus preparation in the presence and in the absence of immune serum, and it is therefore of interest to investigate whether the D_0 method can be used for the same purpose. Decimal dilutions of a rabbit yellow fever hyperimmune serum were mixed with equal volumes of a suspension of mouse brain infected with the 17D strain of yellow fever virus, and the mixtures were inoculated immediately into groups of 11–13 mice by the intracerebral route. The reciprocal survival times in the separate groups were plotted against serum dilution, with the result shown in Fig. 6. With the lowest dilution of serum (10^{-1}) all the virus was neutralized, giving a reciprocal survival time of

zero ; over the 10^{-2}–10^{-4} range of dilutions the reciprocal survival time increased progressively, and between 10^{-4} and 10^{-6} did not differ from the mean value obtained from 2 control groups inoculated with virus and normal serum at the beginning and at the end of the inoculations of the test group. The rising part of the curve thus represents the amount of virus neutralized by varying amounts of antiserum, and is also shown in Fig. 6 in its more familiar form, obtained by transforming the regression coefficient as described above and subtracting the ordinate from the control values. The results thus give a preliminary indication that the D_0 method can be used as the basis of a single-dilution neutralization test and is worthy of further investigation for this purpose.

DISCUSSION

Gard (1940) stated that " theoretically one should expect the (reciprocal survival time) curves to deviate from a straight line in the vicinity of $1/T = 0$ and approach the abscissa asymptotically. The distribution of points in that part of the diagram indicates that this is actually the case ", although the figure given lends little support to this and he has previously pointed out that replicate curves give an unambiguous abscissa intercept of 0·1 minimal infective dose. In the present work, in which more than 100 dose-response curves have been studied, an asymptotic approach has never been observed. The only deviation which may occur is an occasional increase in steepness at high dilutions, due to the survival of animals inoculated with a small amount of virus which develop symptoms and recover.

The use of the incubation period (but not the reciprocal) was suggested by Bryan and Beard (1941) for titrating antibody to rabbit papilloma virus, but the method does not seem to have come into general use. The results obtained in the present work with the 17D strain of yellow fever virus, although only of a preliminary nature, indicate that it should be possible to design a valid neutralization test on the basis of the D_0 single-dilution method which should be more accurate and less cumbersome than the LD50 methods which are standard practice at the present time.

The essential weakness of the LD50 method of titration of viruses is that it rejects most of the information content of the titration procedure. The survival time and its reciprocal are continuous in the mathematical sense, in that they may assume an infinitely great number of values, whereas if only occurrence or non-occurrence of death is recorded the information contained in the survival time is rejected in favour of the more limited information obtained from a non-continuous or quantal response, and the linear response is transformed into the much less convenient sigmoid mortality curve. In practice mice cannot conveniently be subjected to continuous observation, and deaths are commonly recorded to the nearest half-day. As death from infection with a constant dose of virus may occur over a range of 3–4 days, each animal may yield 9 responses, *i.e.* death after 8 possible periods measured in half-days, or indefinite survival. In general, the number of responses which n animals can yield is $n(2t + 1)$, where t is the duration of the symptom period in days, whereas if death or survival is taken as the response, n mice can only yield $n + 1$ responses, *i.e.* the death of successive numbers of mice from zero to n. This disadvantage, together with the practical disadvantage that several virus dilutions must be

inoculated, the responses from which cannot be used in evaluating the LD50, would seem to indicate that the LD50 is not the method of choice for titrating viruses, although it is so firmly established that it will undoubtedly be used for many years to come.

SUMMARY

The reciprocal survival time of mice infected intracerebrally with neurovaccinia, ectromelia, dengue I, rabies and certain other neurotropic viruses has a linear regression upon the logarithm of the dilution of virus in the infecting dose. The dose-response curves thus obtained have an unambiguous abscissa intercept which corresponds to the dose of virus causing zero mortality (D_0). The regression coefficient of the dose-response curve is constant and characteristic of the particular virus. The mean reciprocal survival time of a group of mice inoculated with a single dilution of virus divided by the regression coefficient gives the titre of the preparation immediately in D_0 units. The D_0 bears a fixed relationship to the LD50, and in the case of neurovaccinia the LD50 is equivalent to 40 D_0. The method of single-dilution titration in D_0 units facilitates the carrying out of procedures in which serial titrations are required, such as virus growth curves and thermal inactivation curves, and has many advantages over the usual method of LD50 titration. The D_0 method can also be used for the titration of yellow fever neutralizing antibody and for the determination of antiviral chemotherapeutic activity.

REFERENCES

BAUER, D. J.—(1958) *Brit. J. exp. Path.*, **39**, 480.
Idem AND BRADLEY, P. L.—(1956) *Ibid.*, **37**, 447.
BRYAN, W. R. AND BEARD, J. W.—(1941) *J. infect. Dis.*, **68**, 133.
GARD, S.—(1940) *J. exp. Med.*, **72**, 69.
REED, L. J. AND MUENCH, H.—(1938) *Amer. J. Hyg.*, **27**, 493.
THOMPSON, W. R.—(1947) *Bact. Rev.*, **11**, 115.
TREVAN, J. W.—(1927) *Proc. roy. Soc. B.*, **101**, 483.

CORTISONE-INDUCED RENAL CHANGES IN THE RABBIT: A MICRODISSECTION STUDY

THELMA J. BAXTER

From the Department of Pathology, University of Melbourne, Melbourne, Australia

Received for publication August 31, 1959

TISSUE changes following adrenal corticoid administration are readily demonstrable in the experimental animal particularly when large doses are used. During some experiments, specifically directed to the study of cortisone effects on bone in the rabbit and rat on special "rachitic" diets, definite changes occurring consistently in the kidneys were noted. Simple histological examination showed distinctive changes in several parts of the nephron. The usual difficulties in precise determination of site and nature of these made a study by microdissection desirable.

MATERIALS AND METHODS

Twelve young actively growing rabbits were taken, 6 being given an experimental diet and 6 controls a normal diet. Three from each group were treated with cortisone, the remainder being used as controls. They were killed at intervals of 4, 8 and 12 days. The experimental diet was that used by Steenbock and Black (1925) known as "Ration 2965" and consisted of 76 per cent maize meal, 20 per cent wheat gluten, 1 per cent sodium chloride but cod liver oil was substituted for the 3 per cent calcium carbonate as prescribed.

Cortisone was administered as a 20 mg./ml. solution made up as follows: Cortisone, 6 g.; normal saline, 300 ml.; benzyl alcohol, 2 ml.; Tween 80, 10 drops.

A dose of 20 mg./kg. was injected 6 times weekly subcutaneously into the thigh. The normal diet consisted of Barastoc pellets (a local proprietary food), greens and water.

Experimental method

The kidneys were fixed in formol saline and appropriate portions were removed for histological examination; results determined the selection of kidneys examined by microdissection. Controls were those on normal diets and an animal on the Steenbock-Black diet for 12 days without cortisone. Wedges of renal tissue were incubated for 48 hr. at 19° in concentrated hydrochloric acid, following the technique of Oliver, MacDowell and Tracy (1951).

Apart from the control organs, the kidney from the rabbit on cortisone for 12 days with normal diet and the kidneys of animals treated with cortisone on the Steenbock-Black diet were selected for microdissection. Kidney tissue obtained after cortisone administration for 12 days was at first incubated at 19° for 48 hr. but was found to become very friable and disintegrated often after only a short period, presumably due to the effect of cortisone on the connective tissue. Succeeding macerations were carried out (satisfactorily) at a temperature of 15° and for 48 hr.

At the end of maceration the material was dissected by hand under a stereoscopic microscope, working at magnifications of 50 and 100. Photographs were obtained of material removed from the dissecting dish and mounted in a drop of water on a glass slide. Toluidin blue and Sudan III were used but, for the most part, photographs are of unstained material, made with a Leica camera and adapter using 35 mm. Ilford micro neg. Pan film. Observations were also made and photographs obtained using polarized light.

RESULTS

Tables I and II show the effects of cortisone on the weight of the animals.

TABLE I.—*Weight Changes (g) on Various Days in Rabbits Fed on Normal diet*

	Control animals			Cortisone-treated animals		
Days	(1)	(2)	(3)	(1)	(2)	(3)
0	780	820	582	992	870	598
4	854	896	634	1110	974	700
8	—	996	702	—	946	684
12	—	—	772	—	—	602

TABLE II.—*Weight Changes (g) on Various Days in Rabbits Fed on a Steenbock-Black Rachitic Diet Supplemented by Cod Liver Oil*

	Untreated animals			Cortisone-treated animals		
Days	(1)	(2)	(3)	(1)	(2)	(3)
0	660	916	880	1020	1158	1198
4	606	780	—	1036	1159	1090
8	—	784	740	—	972	886
12	—	—	720	—	—	760

Gross examination showed control kidneys to be normal but enlargement and pallor was common to all treated cases. With the special diet after 4 days of cortisone administration, swelling was minimal but there was a slight area of haemorrhage between cortex and medulla. On the 8th day the organ was swollen and pale with more obvious diffuse haemorrhage at the cortico-medullary junction. By the 12th day gross swelling of the kidneys was present in animals on both the normal and Steenbock-Black (S-B) diets; increase in diameter was approximately one-quarter. The kidneys of animals on the normal diet showed a good demarcation between cortex and medulla but in those on S-B diet cortico-medullary demarcation was less clear; the cortex was irregular with a granular surface; the tissue was rather more translucent than in the controls.

Histological Examination

The control kidneys (both diets) were shown to be histologically normal.

Cortisone and normal diet

Under low magnification, the kidneys after 4 days, apart from some vascular engorgement (more pronounced in the medulla), did not differ significantly from the controls.

The 8-day kidney, except for a narrow segment (corresponding to the cortico-medullary zone), where there was gross dilatation of tubular elements, appeared normal; the area mentioned extended, in some parts, deeply into the medulla.

After 12 days there was vascular engorgement and some scattered haemorrhage. Glomeruli showed some variation in size but, in general, the capsule was well filled by an engorged tuft. Tubular dilatation was widespread, somewhat more obvious in the cortical region where, owing to cellular change, it was often difficult

to distinguish different parts of the tubule. The cells were paler and more translucent than normal and the lumina contained acidophil material. Small casts were present in both proximal and distal tubules.

Cortisone and S-B diet

After 4 days the kidneys were similar to those from the previous group, vascular engorgement being prominent. The 8-day kidney was engorged, slightly haemorrhagic and showed obvious tubular dilatation. The degree of change resembled that seen in the previous group at 12 days.

By 12 days there was gross dilatation of tubules (Fig. 1 and 2) ; in the cortical regions this occurred in areas which alternated with normal zones but there was a well-defined affected band at the cortico-medullary junction. The glomeruli generally were enlarged (Fig. 3 and 4), the tufts being slightly larger than usual, showing considerable congestion as well as an increase in the subcapsular space, that is to say, there was definite distension of Bowman's capsule. The proximal convoluted tubules varied from normal to those showing dilatation with some flattening of the cells. The brush border was still present and there was no significant cytoplasmic or nuclear change. The loops of Henle were dilated, the cells being flattened ; there were scattered cystic dilatations.

The distal convoluted tubules were dilated. Throughout, the normal tubules were intermingled with those showing considerable dilatation.

Both the connecting and collecting tubules showed relatively uniform enlargement though, apart from some slight flattening, significant cellular change was not demonstrable. The terminal part of the collecting tubule was normal except that the nuclei were further from the base of the cell than in the control kidney.

Localized cysts were round, ovoid or elongate in form and scattered irregularly in the deeper part of the cortex. Their origin was not clear from the sections but the lining cells suggested that some at least arose from some part of the tubule.

Capillary engorgement and areas of haemorrhage were more obvious than in the previous specimen, and " oedematous " connective tissue surrounded some of the larger vessels of arteriolar and small arteriole type.

As in the 12-day kidney (cortisone with normal diet) there were casts in the proximal and distal tubules (Fig. 5). With PAS stain the casts were fuchsinophilic, staining as intensely as the basement membrane (Fig. 6). Both Perl's reaction for ferric salts, modified according to Lillie (1948) and the Prussian blue reaction gave negative results but the benzidine test proved positive for the presence of haemoglobin.

Frozen sections stained with Sudan red showed little or no fat in the earlier specimens but by the 12th day (with both normal and S-B diets) lipid was demonstrable in the tubule cells. With the cortisone and S-B diet, lipid was especially obvious at the cortico-medullary junction, whilst on a normal diet there was a more even distribution ; however, it was abundant in the inner medullary region.

Microdissection

Small portions from various areas in the kidneys from animals fed on both normal and S-B diets for 12 days were microdissected. No abnormality was observed.

Specimens from cortisone-treated animals showed connective tissue friability

which demanded modifications of maceration technique. When this was adequately dealt with nephron changes were found, the degree of change being related to the length of the experiment. For simplification and comparison of pathological change the various components of the nephron are described separately.

Glomeruli of the normal organ were uniform, measuring 105 μ to 115 μ but after cortisone administration they showed variations in form. At the 4th day, there was an occasional slight increase in subcapsular space (Fig. 7). By the 12th day (normal diet) slight enlargement was associated with a prominent subcapsular space (Fig. 14). With the S-B diet changes of slight degree were present at 8 days ; by the 12th day, however, the glomeruli varied in diameter, shape and density of the tuft, being either round or slightly ovoid in shape and ranged in diameter 75 μ–240 μ. In the smaller glomeruli, tufts were very dense but in the larger ones were pale-staining and " transparent ".

The proximal convoluted tubules were regular in type in the control animals but in the cortisone-treated animals on both normal and special diets changes were widespread and, after 4 days, were evident in all kidneys. At 4 days, change of slight degree was confined to the terminal portion of the proximal convoluted tubule, loop of Henle and distal convoluted tubule. In the late stages dilatation was gross, dilated areas alternating with apparently normal regions (Fig. 8, 10, 11, 20, 21 and 22). In some zones the tubules appeared flattened, " acellular " and hyaline in character (Fig. 18).

The proximal convoluted tubule of the control animal measured 35–45 μ in diameter but with cortisone, dilatation ranged from slight to extreme, the largest diameter registered in this tubule being 230 μ. Frequently this segment had a " scalloped " effect, dilatations alternating with constrictions. In one proximal convoluted tubule the minimum diameter was 47 μ and the maximum 94 μ. At all stages with the normal diet and in the early stages, up to 8 days on the S-B diet, the terminal part of the proximal convoluted tubule was straight and unkinked (Fig. 15 and 17) ; however, by 12 days (S-B diet) there was a kinking in the terminal region so that it had a definite spiral character—almost corkscrew in nature (Fig. 12, 13 and 16). In unstained material the cells contained intensely dark granules so that the terminal proximal convoluted tubule stood out prominently.

When stained with Sudan red the cells of the proximal tubule, and those extending to the loop of Henle, showed numerous small droplets of lipid material which at first were fine but later became coarser. With polarized light some material was anisotropic (Fig. 19). The nephrons came from the cortico-medullary zone of the kidney in which this material had been shown in sections.

Intratubular material of two kinds occurring either separately or in association, found by the 8th day of S-B diet (Fig. 15) was more prominent by the 12th day. One type contained cells, desquamated epithelial cells and some erythrocytes ; haemoglobin was present (Fig. 14 and 15). The second material was homogeneous, staining lightly with haematoxylin. Under polarized light this was present particularly in the terminal part of the proximal tubule (Fig. 12 and 13), and in the loop of Henle adjacent to the cortico-medullary junction.

In the ascending limb of the loop of Henle, apart from slight dilatation, change was observed only in the case of the kidney after 12 days' cortisone. Here the dark granular material seen in the cells of the terminal proximal convoluted

tubules of the other kidneys, was found in the ascending limb, which appeared very dark, almost black in colour, and similar in appearance to the distal convoluted tubule.

The distal convoluted tubule and collecting tubules were somewhat dilated in all kidneys of treated animals ; intratubular material was present here (Fig. 23 and 24). Dilatation was progressive, being maximal in the 12-day kidneys (S-B diet). Variation in diameter of the collecting tubules in the vicinity of the glomeruli ranged from 28–94 μ (control animal : 28–38 μ). Some polarizable lipid was present here also.

In correlating the microdissection with the histological appearances, only occasional cysts were found and these were relatively small (Fig. 9). The dilatation of the terminal part of the proximal convoluted tubule, the ascending limb of the loop of Henle and collecting tubules corresponded to the generally dilated area at the cortico-medullary junction.

Little change was observed in the vessels apart from engorgement. A notable feature was the presence of finely divided lipid in many of the small vessels—some of this being anisotropic. Lipid was also found in some small clumps of connective tissue which had survived the acid maceration. In places, the connective tissue contained a dark sticky substance by means of which the tissue remained firmly attached to the tubules.

DISCUSSION

Observations on cortisone administration in both clinical and experimental work have shown its effect is inhibitory to growth and metabolism. It is anti-mitotic, since it inhibits the pre-prophase stage of mitosis when injected or applied to the epidermis of the mouse (Green and Ghadially, 1951) and in this role plays an important part in the " anti-allergic, anti-inflammatory and anti-repair " effects observed. It is anti-metabolic as it affects protein metabolism (Sprague, 1951) and carbohydrate tolerance (Ragan, Howes, Plotz, Meyer and Blunt, 1949). These effects have been investigated mainly in connective tissues, both in simple reticular and fibrous tissues, as well as in cartilage and bone (Sissons and Hadfield, 1951 ; Sissons, 1955, 1956).

Resorption of bone following cortisone administration occurs readily in the rabbit, but not in the rat (Storey, 1957, 1958). However, gross resorptive change does occur in the rat when the Ca/P ratio in the diet is disturbed (Storey, 1960). In this special circumstance (using the S-B diet) it was found that even more extreme changes (including gross rarefaction and spontaneous fractures) are observed in the rabbit and it was during these experiments that the renal changes were noted in the rabbit and investigated by microdissection.

The main feature is a general enlargement of the organ. This is clearly due to swelling (probably oedema) of the connective tissues as well as increased vascularity but there is also distension and enlargement of the glomeruli and tubules.

Swelling of the glomeruli is due partly to enlargement of the tuft but also to increase in the subcapsular space. There is irregular dilatation of the proximal convoluted tubule and an obvious kinking in the terminal region, dilatation of the loop of Henle and some enlargement of the connecting tubules. Altogether there is a general dilatation of the nephron.

Other changes include irregularly distributed zones of increased fragility ; these are apparent in dissolution of some areas during examination. The more

rapid maceration of the specimens was interpreted as due to the connective tissue changes resulting from the cortisone treatment.

Cytological changes include the accumulation of fat in the tubule cells and the swelling of many of them so that they were more translucent than usual. It has been shown that, in rabbits, cortisone administration has produced a gross degree of lipemia (Rich, Cochran and McGoon, 1951), hypercholesterolemia and a depression of the phospholipid-cholesterol ratio (Oppenheim and Bruger, 1952), these may be related to depositions of the fat in the glomeruli and tubule cells. Oliver (1950) noted, in " fatty kidneys ", that there was no regularity in deposition of fat, but in the present experiments, particularly in the 12-day kidney (cortisone and S-B diet) the greatest concentration was at the cortico-medullary junction. Fatty glomerular and tubular lesions with cortisone administration to rabbits were observed by Wilens and Stumpf (1955) who attributed the changes to the blood lipids inducing a glomerular stasis, so relating this condition to the stasis observed in the capillaries of the intestinal tract in alimentary lipemia. They also observed debris within the tubules similar to that described here.

The effect of cortisone may be either direct or indirect. It has been shown that the re-absorptive capacity of the renal tubules for phosphates is depressed by cortisone (Roberts and Pitts, 1953). They also noted that phosphate depression is associated with a depletion of intracellular stores of phosphate and bone demineralization. On the other hand, cortisone may cause also water and electrolyte retention with oedema (Sprague, 1951 ; Sprague, *et al.*, 1950 ; Roberts and Pitts, 1952 ; Hills, Chalmers, Webster and Rosenthal, 1953 ; Walser, Seldin and Burnett, 1955 ; Davis, Lindsay and Southworth, 1952 ; Davis and Howell, 1953*a* and 1953*b* ; Davis, Howell and Southworth, 1953 ; Berliner, 1952), and this has been shown to be so particularly in rabbits (Aikawa, 1953). This worker noted that the effect in different circumstances (for example, dosage and period of administration) was to give opposing results, but, in general, the level of water exchange is raised (renal plasma flow and glomerular filtration rate are increased by cortisone).

Berliner (1952) also noted that the sodium re-absorption is accompanied by chloride re-absorption and the excretion of potassium. These ionic imbalances may well produce indirect effects on the tubules, especially since part of them is " involved in the hormonal regulation of sodium metabolism " (Hartroft and Hartroft, 1953). The disturbance of ionic balance is associated with depletion of the body's store of potassium. This occurs partly by faecal electrolyte excretion (Davis, Lindsay and Southworth, 1952) and by urinary excretion (Sprague, 1951 ; Berliner 1952 ; Aikawa, 1953).

There is clear evidence that potassium depletion is one factor responsible for tubular change. Renal hypertrophy has been noted as a prominent feature in potassium deficiency (Schrader, Prickett and Salmon, 1937 ; Follis, Orent-Keiles and McCollum, 1942 ; Kornberg and Endicott, 1946 ; Danford and Herrin, 1951 ; Milne and Muehrcke, 1957).

On potassium deficient diets, rats show tubular casts, irregular dilatation of collecting and convoluted tubules and " fraying " of the epithelium of distal collecting tubules (Kornberg and Endicott, 1946 ; Schrader, Prickett and Salmon, 1937). Spargo (1954) observed a granular cytoplasm in the collecting tubule cells and the change may progress to a degree designated " necrosis " (MacPherson and Pearce, 1957 ; Milne and Muehrcke, 1957), the degree of change being related to the duration of the experiment and the degree of depletion.

EXPLANATION OF PLATES

FIG. 1–5.—Photomicrographs of sections stained with haematoxylin and eosin.

FIG. 6.—Stained with PAS.

FIG. 7–24.—Photomicrographs of microdissections.

FIG. 1.—Control kidney (S-B diet without cortisone). Structure normal. ×30.

FIG. 2.—Kidney (S-B diet, 12 days' cortisone) showing dilatations of tubules and enlargement of glomeruli. ×30.

FIG. 3.—Control kidney (S-B diet without cortisone) showing normal glomerulus and tubules. ×500.

FIG. 4.—Kidney (S-B diet, 12 days' cortisone) showing enlargement of glomerular tuft and subcapsular space. ×500.

FIG. 5.—Kidney (12 days' S-B diet and cortisone) showing a group of erythrocytes in the lumen of the tubule. ×175.

FIG. 6.—Kidney (12 days' S-B diet and cortisone) stained with PAS showing a mass of desquamated cells and lipid in the lumen of a tubule. ×175.

FIG. 7.—Proximal convoluted tubule and glomerulus showing large subcapsular space. Most of the glomeruli were normal. (S-B diet, 4 days' cortisone.) ×100.

FIG. 8.—Glomerulus attached to grossly dilated proximal convoluted tubule. Blood vessels from the glomerulus can be seen coursing over the tubular surface. Average diameter of tubule, 150 μ (S-B diet, 12 days' cortisone). ×80.

FIG. 9.—Small cystic glomerulus with prominent subcapsular space. Diameters 225 μ × 160 μ. (S-B diet, 8 days' cortisone.) ×150.

FIG. 10.—Portion of proximal convoluted tubule showing gross dilatation and disruption of the basement membrane. (S-B diet, 8 days' cortisone.) ×150.

FIG. 11.—Cystic type dilatation of short length of proximal convoluted tubule. The dark material within the lumen is clearly visible. Maximum diameter = 188 μ. (S-B diet, 12 days' cortisone.) ×160.

FIG. 12.—Complete proximal convoluted tubule showing spiral twisting of normally straight terminal segment and intensely dark appearance of this region. (S-B diet, 12 days' cortisone.) ×30.

FIG. 13.—Terminal segment of proximal convoluted tubule shown in Fig. 12. ×100.

FIG. 14.—Portion of proximal convoluted tubule near glomerulus, to show enlarged subcapsular space and the dark aggregations within the tubule. (Normal diet, 12 days' cortisone.) ×100.

FIG. 15.—Terminal portion of proximal convoluted tubule showing dark material deposited within the lumen as far as the loop of Henle. (S-B diet, 8 days' cortisone.) ×100.

FIG. 16.—Terminal segment of proximal convoluted tubule to show the " corkscrew " type of spiral kinking so frequently seen in kidney. (S-B diet, 12 days' cortisone.) ×500.

FIG. 17.—Terminal portion of proximal convoluted tubule to show absence of kinking and presence of numerous small masses of intra- and extracellular material. (Normal diet, 12 days' cortisone.) ×500.

FIG. 18.—Three lengths of terminal proximal convoluted tubule. Two are " acellular " and dilated, the other is not dilated but has intensely dark cellular contents. Diameters: 47–56 μ (dilated tubules), 23–25 μ (undilated tubule). (S-B diet, 12 days' cortisone.) ×100.

FIG. 19.—Portions of proximal convoluted tubule photographed with crossed polars to show deposition of lipid. The larger lipid deposits shown are situated in connective tissue. (S-B diet, 12 days' cortisone.) ×100.

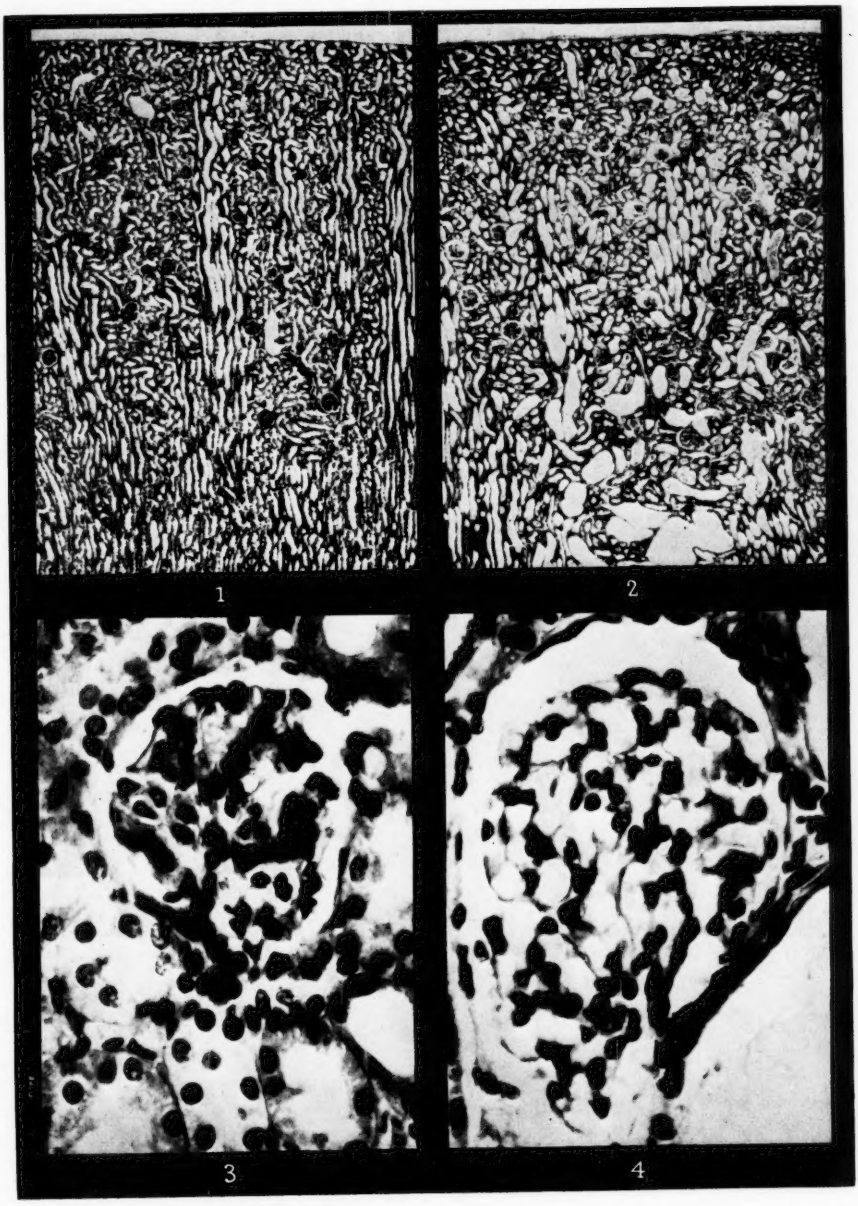
FIG. 20.—Blunt, dilated, localized cystic dilatation of proximal convoluted tubule. Dark bodies within the tubule are clearly seen. (S-B diet, 8 days' cortisone.) ×160.

FIG. 21.—Grossly dilated proximal convoluted tubule compared with normal. The dilated tubule contained a dark mass in the lumen. (S-B diet, 12 day's cortisone.) ×250.

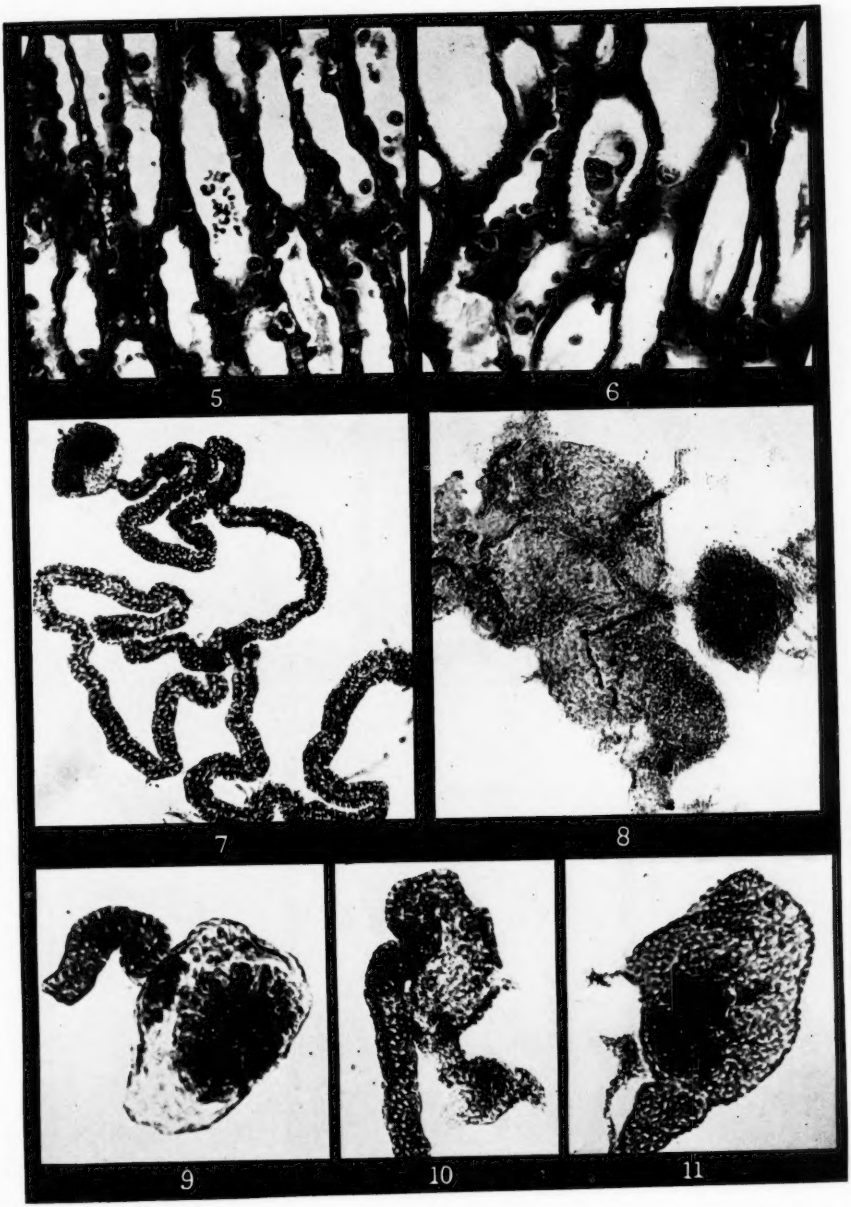
FIG. 22.—Grossly distorted portion of proximal convoluted tubule, illustrating eccentric cystic development. (S-B diet, 12 days' cortisone.). ×90.

FIG. 23.—Large pale collecting tubule showing a local cystic dilatation. (S-B diet, 8 days' cortisone.) ×250.

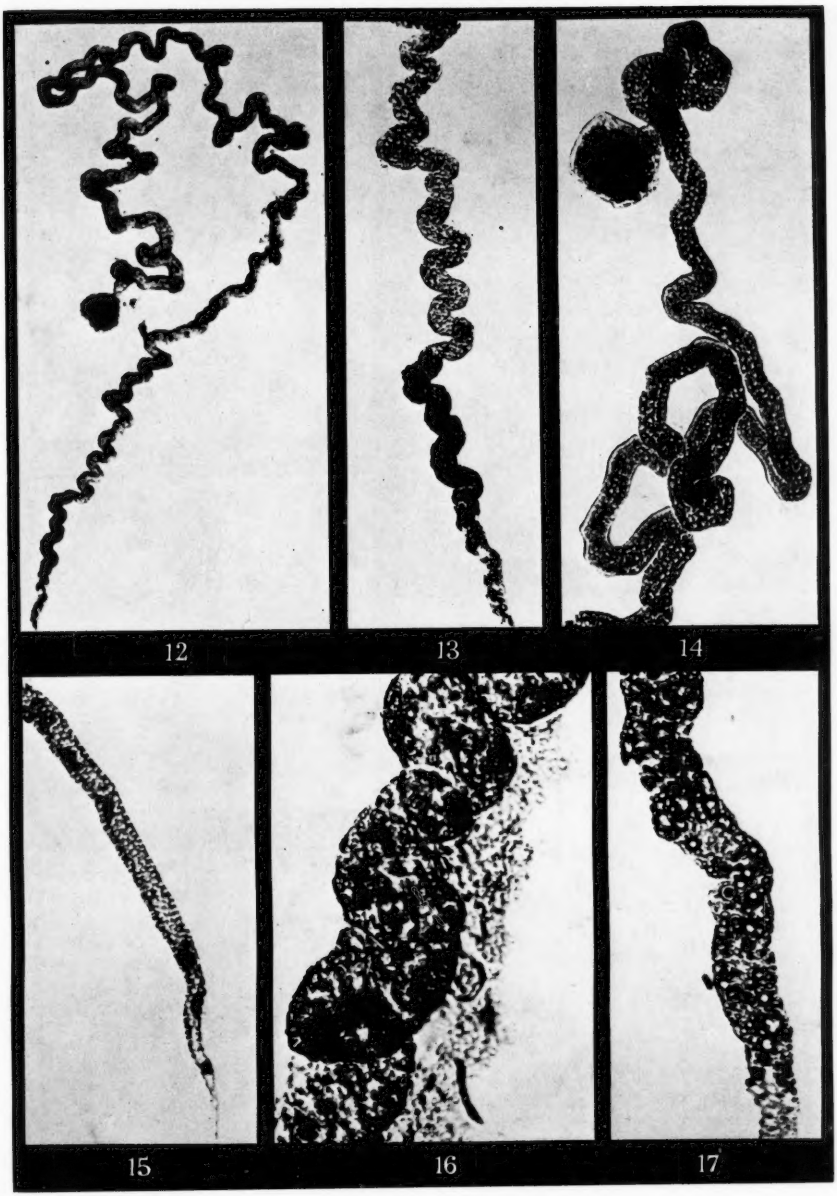
FIG. 24.—Two portions of collecting tubule, one grossly dilated and almost segmental in appearance, the other normal in size. No attempt was made to stretch these segments as the material was friable and fragmented when touched with needles. Large tubule: 85 μ maximum, 38 μ minimum. Small tubule: 38 μ average diameter. (S-B diet, 8 days' cortisone.) ×110.



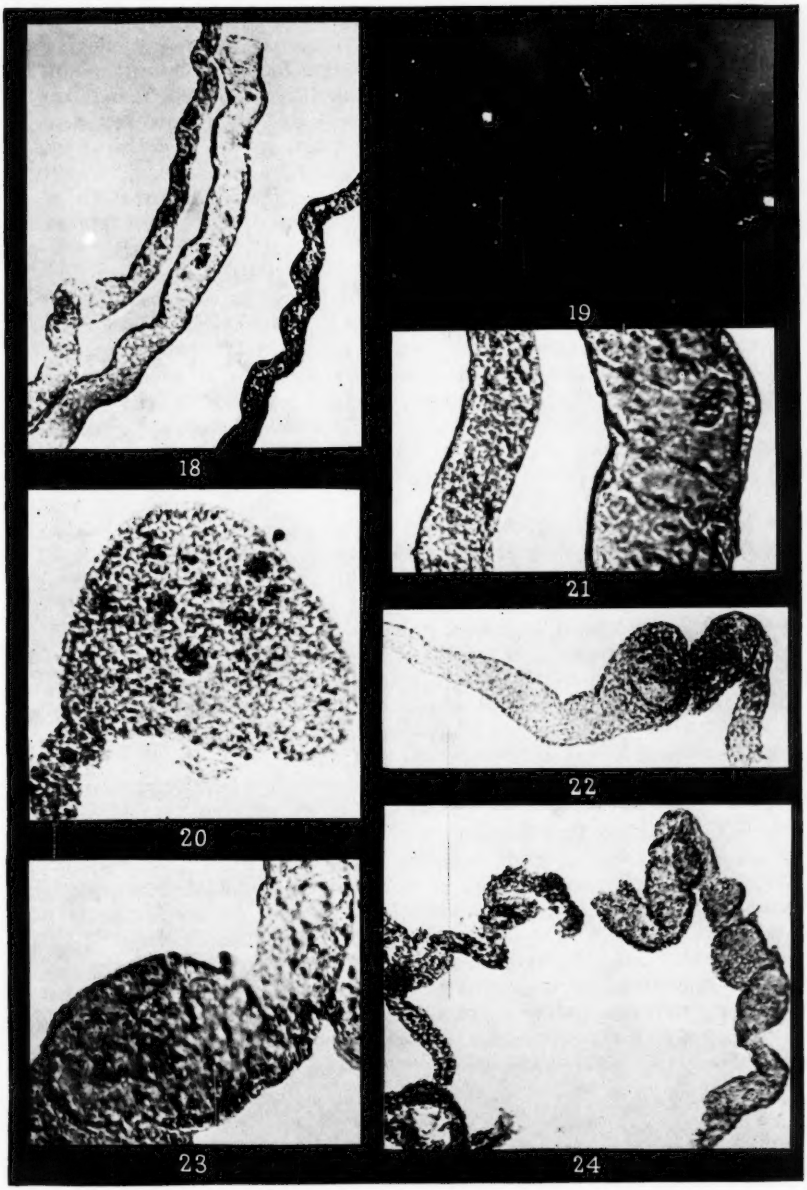
Baxter.



Baxter.



Baxter.



Baxter.

Rats on a sodium-potassium diet ratio of 2 : 1 to 20 : 1 showed tubular dilatation and with a ratio of 200 : 1 a gross degree of change (" necrosis ") occurred (Meyer, Grunert, Zepplin, Grummer, Bohstedt and Phillips, 1950). Oliver *et al.*, (1957) demonstrated a zone of involvement—" the outer zone of the medulla "—where tubular dilatation was maximal. There was thickening in the lower third of the proximal convoluted tubule with a progression of the lesion to its terminal segment, hyperplasia of the epithelial cells and accumulation of intracellular granules.

In relation to the present experiments it is noteworthy that the potassium requirements of the 3- to 4-week-old rabbit is " several times that the of rat " (Wooley, 1954).

On the other hand, changes are produced by adrenal steroids. Renal enlargement, with hyperplasia of convoluted tubules, follows administration of desoxycorticosterone acetate (Ludden, Krueger and Wright, 1941 ; Durlacher, Darrow and Winternitz, 1942 ; Darrow and Miller, 1942 ; Deane, Shaw and Greep, 1948). There is an increase of sodium and a diminution of potassium in muscle (Miller and Darrow, 1941 ; Ferrebee, Parker, Carnes, Gerity, Atchley and Loeb, 1941) and an electrolyte shift occurs with cortisone administration (Deane *et al.*, 1948). A cortical adenoma of the adrenal gland in man was associated (as shown by renal biopsy) with a " diffuse vacuolar change " in tubular epithelium (Conn, 1955).

Thus the changes occurring in the kidneys in potassium depletion closely resemble those following cortical steroid action (Darrow and Miller, 1942 ; Durlacher *et al.*, 1942 ; Follis *et al.*, 1942 ; Darmady and Stranack, 1957). In this regard, Henrikson (1951), working with rats, noted that one-quarter to one-third of the body potassium was lost in the first 9 days of treatment, either by using a diet of 0·01 per cent potassium or by the injection of cortisone.

Ionic changes are produced in several ways and the adrenal gland is " only one of a number of factors which influence electrolyte metabolism " (Sayers, 1950) ; *i.e.* renal activity is itself an important factor.

The fluid accumulation, which is a prominent feature of the renal tissue, is probably to be correlated with general oedema. The occurrence of fat in the tubule cells in the present experiment is noteworthy since most observers record the absence of lipid from the kidneys in experimental hypokalaemia. The precise causes of the changes are thus still uncertain. Even in the experiments where primary potassium depletion is induced there may be secondary hormonal effects. Despite these difficulties the morphological changes demonstrated provide a foundation on which further physiological studies may be built. The relation of these changes to those in other animals or man, particularly in view of metabolic differences between species (Wooley, 1954), is necessarily uncertain. The doses employed here are beyond the physiological range ; at the same time gross changes using high doses may indicate the presence of minor changes at a submicroscopic level not demonstrable morphologically when smaller dosage is employed.

SUMMARY

When cortisone is administered to rabbits, general changes occur affecting mainly the connective tissues. If the animals are placed on a " rachitic " diet the changes are much more gross.

In such cases obvious changes are apparent in the parenchyma (as well as the connective tissues) of the kidneys.

These have been studied by microdissection. They include enlargement of glomeruli with increase of capsular space and swelling of the tuft. There is dilatation, diffuse or localized, in the tubules and tubular casts of red blood corpuscles and pigmented and fatty material occur. Fat is found in the glomeruli and tubules. Small cysts also occur in the cortex and are due to both glomerular or tubular change.

It has not yet been possible to determine whether the observed changes were due to a direct action of cortisone on the renal tissue, or to altered ionic balance.

My grateful thanks to Dr. E. Storey of this department for making available to me the rabbit kidneys from some of his experimental work, and for the data contained in Tables I and II. My thanks are due to the Upjohn Company, Kalamazoo, Michigan, U.S.A. who presented the cortisone acetate used in this work.

REFERENCES

AIKAWA, J. K.—(1953) *Proc. Soc. exp. Biol. N.Y.*, **82**, 105.
BERLINER, R. W.—(1952) *Fed. Proc.*, **11**, 695.
CONN, J. W.—(1955) *J. Lab. clin. Med.*, **45**, 3.
DANFORD, H. G. AND HERRIN, R. C.—(1951) *Amer. J. Physiol.*, **165**, 128.
DARMADY, E. M. AND STRANACK, F.—(1957) *Brit. med. Bull.*, **13**, 21.
DARROW, D. C. AND MILLER, H. C.—(1942) *J. clin. Invest.*, **21**, 601.
DAVIS, J. O., LINDSAY, A. E. AND SOUTHWORTH, J. L.—(1952) *Johns Hopk. Hosp. Bull.*, **90**, 64.
Idem AND HOWELL, D. S.—(1953*a*) *Endocrinology*, **53**, 653.—(1953*b*) *Circulation*, **1**, 171.
Iidem, AND SOUTHWORTH, J. L.—(1953) *Ibid.*, **1**, 260.
DEANE, H. W., SHAW, J. H. AND GREEP, R. O.—(1948) *Endocrinology*, **43**, 133.
DURLACHER, S. H., DARROW, D. C. AND WINTERNITZ, M. C.—(1942) *Amer. J. Physiol.*, **136**, 346.
FERREBEE, J. W., PARKER, D., CARNES, W. H., GERITY, M. K., ATCHLEY, D. W. AND LOEB, R. F.—(1941) *Ibid.*, **135**, 230.
FOLLIS, R. H., ORENT-KEILES, E. AND MCCOLLUM, E. V.—(1942) *Amer. J. Path.*, **18**, 29.
GREEN, H. N. AND GHADIALLY, F. N.—(1951) *Brit. med. J.*, i, 496.
HARTROFT, P. M. AND HARTROFT, W. S.—(1953) *J. exp. Med.*, **97**, 415.
HENRIKSON, H. W.—(1951) *Amer. J. Physiol.*, **164**, 263.
HILLS, A. G., CHALMERS, T. M., WEBSTER, G. D. Jr. AND ROSENTHAL, O.—(1953) *J. clin. Invest.*, **32**, 1236.
KORNBERG, A. AND ENDICOTT, K. M.—(1946) *Amer. J. Physiol.*, **145**, 291.
LILLIE, R. D.—(1948) ' Histopathologic Technic '. Philadelphia (The Blackiston Co.), p. 124.
LUDDEN, J. B., KRUEGER, E. AND WRIGHT, I. S.—(1941) *Endocrinology*, **28**, 619.
MACPHERSON, C. R. AND PEARSE, A. G. E.—(1957) *Brit. med. Bull.*, **13**, 19.
MEYER, J. H., GRUNERT, R. R., ZEPPLIN, M. T., GRUMMER, R. H., BOHSTEDT, G. AND PHILLIPS, P. H.—(1950) *Amer. J. Physiol.*, **162**, 182.
MILLER, H. C. AND DARROW, D. C.—(1941) *Ibid.*, **132**, 801.
MILNE, M. D. AND MUEHRCKE, R. C.—(1957) *Brit. med. Bull.*, **13**, 15.
OLIVER, J.—(1950) *Amer. J. Med.*, **9**, 88.
Idem, MACDOWELL, M. AND TRACY, A.—(1951) *J. clin. Invest.*, **30**, 1305.
Idem, MACDOWELL, M., WELT, L. G., HOLLIDAY, M. A., HOLLANDER, W. Jr., WINTERS, R. W., WILLIAMS, T. F. AND SEGAR, W. E.—(1957) *J. exp. Med.*, **106**, 563.

OPPENHEIM, E. AND BRUGER, M.—(1952) *Circulation*, **6**, 470.
RAGAN, C., HOWES, E. L., PLOTZ, C. M., MEYER, K. AND BLUNT, J. W.—(1949) *Proc. Soc. exp. Biol. N.Y.*, **72**, 718
RICH, A. R., COCHRAN, T. H. AND MCGOON, D. C.—(1951) *Johns Hopk. Hosp. Bull.*, **88**, 101.
ROBERTS, E. K. AND PITTS, R. F.—(1952) *Endocrinology*, **50**, 51.—(1953) *Ibid.*, **52**, 324.
SAYERS, G.—(1950) *Physiol. Rev.*, **30**, 241.
SCHRADER, G. A., PRICKETT, C. O. AND SALMON, W. D.—(1937) *J. Nutr.*, **14**, 85.
SISSONS, H. A.—(1955) *J. Anat. Lond.*, **89**, 69.—(1956) *J. Bone Jt Surg.*, **38**B, 418.
Idem AND HADFIELD, G. J.—(1951) *Brit. J. Surg.*, **39**, 172.
SPARGO, B.—(1954) *J. Lab. clin. Med.*, **43**, 802.
SPRAGUE, R. G.—(1951) *Amer. J. Med.*, **10**, 567.
Idem, POWER, M. H., MASON, H. L., ALBERT, A., MATHIESON, D. R., HENCH, P. S., KENDALL, E. G., SLOCOMB, G. H. AND POLLEY, H. F.—(1950) *Arch. intern. Med.*, **85**, 199.
STEENBOCK, H. AND BLACK, A.—(1925) *J. biol. Chem.*, **64**, 263.
STOREY, E.—(1957) *Aust. N.Z. J. Surg.*, **27**, 19.—(1958) *J. Bone Jt Surg.*, **40**B, 559.—(1960) *Brit. J. exp. Path.* (in the press).
WALSER, M., SELDIN, D. W. AND BURNETT, C. H.—(1955) *Amer. J. Med.*, **18**, 454.
WILENS, S. L. AND STUMPF, H. H.—(1955) *Amer. J. Path.*, **31**, 275.
WOOLEY, J. G.—(1954) *J. Nutr.*, **52**, 39.

THE LOCALIZATION OF LESIONS IN EXPERIMENTAL FOOT-AND-MOUTH DISEASE

H. PLATT

From the Research Institute (Animal Virus Diseases), Pirbright, Surrey

Received for publication September 1, 1959

THE characteristic epithelial lesions of foot-and-mouth disease (FMD) show a striking predilection for certain parts of the integument. In a previous paper (Platt, 1958*a*), it was shown that the sites of lesions in the experimentally infected guinea-pig are the tongue, lips, prepuce, metatarsal pads, plantar surfaces of the feet and, in lactating females, the teats ; small lesions also occur in the skin of the ear and sometimes in the oesophageal mucosa and the perineum. Epidermal lesions do not occur in hairy skin, but in some animals, occasional hair follicles are damaged ; the occurrence of high virus titres in the hairy skin of a proportion of guinea-pigs is presumably related to this follicular damage.

In the present experiments, an attempt has been made by the use of grafting techniques to elucidate some of the possible factors concerned in this characteristic localisation. Epithelium from the predilection sites transplanted to the chest, and conversely, hairy skin from the flank grafted to the tongue and metatarsal pads, underwent considerable alterations in their susceptibility to damage by the virus. The underlying tissue changes responsible for this were then investigated in the light of these findings.

METHODS

Experimental animals.—Albino guinea-pigs, usually 400–600 g. of either sex, from the Institute breeding colony were used in most experiments but where pigmented grafts were needed, pied or parti-coloured guinea-pigs were obtained from 2 dealers. They were kept in wire cages and fed on a pellet diet supplemented with green food, hay and water. Before infection, they were transferred to a room kept at 21°, where they were fed on bran mash.

Grafting techniques.—Anaesthesia was effected with Veterinary Nembutal (0·3–0·5 ml./kg.) supplemented by ether when necessary. In each animal, a pinch graft from either the centre of the metatarsal pad, the dorsum of the tongue near the tip or the epithelium at the preputial orifice, was grafted to the right side of the chest ; alternatively, hairy skin from the flank was grafted to the tongue or the metatarsal pad. Pinch grafts were used throughout since these retain best the characteristics of the donor epithelium (Billingham and Medawar, 1951). In grafting to the chest, the technique of these authors was closely followed and their paper may be consulted for further details. When grafting hairy skin to the tongue, grafts 2–3 mm. in diameter of black skin were inserted into shallow sub-epithelial pockets, the slit entrance of which usually healed in 1–2 days, provided no graft protruded. If, however, the pocket roof was lost before the graft had become vascularized, the latter usually underwent maceration by the saliva. Established grafts gradually broke through to the surface by degeneration of the overlying epithelium but grafts that were too deeply located became encysted and surgical exposure was necessary. Grafts of hairy skin on the metatarsal pad required special treatment, as there was a tendency for pad epithelium to burrow under the graft before it had become vascularized. Grafts were therefore inserted beneath the thick undercut flaps of a Y-shaped incision in the centre of the pad and the flaps were replaced

over the graft, where they gradually degenerated. A simple dressing of Elastoplast was applied round the foot at this point and was readily tolerated provided it was not too tight. Animals with white pads were used and the grafts were of black skin.

Mode of infection.—Guinea-pigs were infected either intraperitoneally or by intradermal injection of the metatarsal pads with a suspension in 0·04 M buffered phosphate (pH 7·6) of freshly drawn vesicular fluid from 18–24 hr lesions in guinea-pigs infected with the virus of FMD (Strain V.1 ; Vallée type O). Unless stated to the contrary, the intradermal route was used. The distribution of lesions remains unaffected by the choice of route, although after intraperitoneal infection the course of the disease is more protracted.

Microscopy and estimation of epithelial mitotic activity.—Guinea-pigs were killed 1–8 days after infection and material for histological examination was fixed in 10 per cent neutral formol-saline. Hairy skin and samples of preputial epithelium for mitotic counts were gently extended on cardboard or cork before fixation. Tissues were dehydrated in alcohol, cleared in chloroform and embedded in paraffin wax. Sections were cut at5–6 μ and stained with haematoxylin and eosin. Serial sections at 25–60 μ intervals were cut through many of the grafts.

The mitotic activities of grafts and their respective tissues of origin in the same animals were compared 6 hr after the administration of colchicine (1 mg./Kg.). Its use minimizes errors associated with the rather low mitotic counts in some types of skin and with possible differences in mitotic duration in different regions. A count of all epithelial mitoses within an arbitrarily demarcated length of epithelium was made, including in hairy skin, those in the follicle necks down to the level of the sebaceous glands. The length of the basal layer in the demarcated sector was then estimated with a map measurer on projected images of the epithelium at $\times$ 200 magnification. The results are calculated as total epithelial mitoses/mm. basal layer/6 hr.

Titration of the virus content of metatarsal pad grafts on the chest.—This was performed 48 hr after intradermal infection of the metatarsal pads when secondary generalization is at its height. Since the prevailing viraemia may give rise to spuriously high tissue virus titres, the blood was removed by perfusion. Guinea-pigs were given Heparin (5 mg. I.M.) and deeply anaesthetized (Nembutal and ether). The right ventricle was incised and the animal was perfused via a cannula in the left ventricle with about a litre of warm saline. After removal of the horny overlay, the grafts were excised and weighed. Since the intercellular fluid of the graft and its adherent dermis might contain residual virus, a weighed sample of the underlying connective tissue was titrated as a control. The samples were ground with a minimum of sand and titrated in serial 10-fold dilutions in 7-day old suckling mice. Each dose was injected into 5 mice among at least 2 litters. Under these conditions titre differences of 1·0 log unit are likely to be significant at the 5 per cent level.

Treatment of metatarsal pad grafts with histamine or Clostridium oedematiens *toxin.*—Histamine (as histamine acid phosphate) was injected intradermally at 1–3 sites near the graft margin, usually in 0·1 ml. saline, 1 day after intradermal infection of the pads. Gentle intermittent suction for 5–10 min. after injection was applied to the grafts with a suction cup to raise local intracapillary pressure. Histamine iontophoresis was performed with 0·17 per cent histamine solution (0·5 per cent histamine acid phosphate) and a current of 1 ma. for 20–40 min. under light Nembutal anaesthesia. *Cl. oedematiens* exotoxin was a freshly prepared solution of a dilution which, on intradermal injection into normal guinea-pigs, gave a well defined erythematous reaction at 48 hr. without central necrosis. A single injection in 0·1 ml. saline was made at the graft margin on the day of infection.

RESULTS

Grafts of hairy skin on the tongue

Grafts of this type, when fully exposed, formed blackish circumscribed areas 1–3 mm. diameter from which occasional black hairs protruded ; there was no pigment spread into the surrounding epithelium (Fig. 1). Microscopically, they were typically of hyperplastic skin, although hair follicles and sebaceous glands were rather reduced in numbers. Basophilia of the graft Malpighian layer was less than in the tongue of which the deep epithelial layers were intensely basophilic.

The grafts had a conspicuous stratum granulosum, while the stratum corneum was as thick as the corresponding layer in the tongue.

Sixteen guinea-pigs grafted on the tongue were infected; in 3, the graft was in the form of a submucosal cyst (Fig. 2). The results 1–2 days after infection are shown in Table I.

TABLE I.—*Extent of Necrosis of Skin Grafts on the Tongue in Experimental FMD*

Route of infection I.D.	Route of infection I.P.	Extent of lesions: Tongue	Extent of lesions: Graft	No. of animals	Duration of exposure of grafts (days)
2	2	Minimal or none	None	4	0*–59
2	—	Severe or moderately severe	None	2	0*
3	1	,,	Predominantly marginal†	4	17–63
3	3	,,	Widespread	6	6–143

* Buried encysted grafts.
† Occasional microscopic foci in the graft interior in two cases.

Exposed hairy skin grafts on the tongue dorsum became susceptible in varying degrees to FMD virus but lesions in the grafts were observed only in guinea-pigs with well marked secondary tongue lesions. The encysted grafts, however, remained unaffected even when the buried graft lay close to severely necrotic tongue epithelium (Fig. 3). In the 10 animals with involvement of both graft and tongue epithelium, the extent of damage varied. In some the margin only was involved and in these instances the graft was clearly less susceptible than the surrounding tongue epithelium. In other cases, however, the grafts were extensively damaged and to a degree varying from necrosis of the upper half of the Malpighian layer to total destruction in some areas (Fig. 4). The depths of the hair follicles were usually spared even in regions where the necrosis was severe.

Grafts of hairy skin on the metatarsal pad

These were black hairless areas, usually 2·5–3·0 mm. in diameter, slightly elevated above the general level of the pad and showing marked pigment spread into the surrounding epithelium (Fig. 5). Microscopically, they displayed considerable hyperplasia and a dense stratum corneum. Nevertheless, they retained most of the native characteristics of hairy skin including hair follicles and sebaceous glands. The hyperplastic epidermis was pierced by follicle shafts containing hair stumps, but actively growing hairs were not seen. The granules of the stratum corneum were coarse and irregular in contrast with the smaller more numerous granules in this layer of the pad epithelium.

The susceptibility of such grafts to damage by the virus was investigated in 7 guinea-pigs infected intraperitoneally 30–202 days after grafting. All developed severe lesions of the metatarsal pads and the grafts within 2–5 days of infection and the susceptibility of the grafts appeared to be similar to that of the metatarsal pads. The most severe destruction was in the hyperplastic epidermis, while the depths of the follicles were usually spared. The lesions consisted of epithelial cell necrosis and leucocytic infiltration similar in type to the lesions produced by the virus in other sites, but vesiculation was not observed and the leucocytic

invasion of the necrotic epithelium was more profuse than in comparable lesions in the surrounding pad epithelium (Fig. 6).

Grafts of tongue epithelium on the chest

These formed sharply circumscribed areas 1–3·5 mm. in diameter overlaid by thick horn which sometimes developed into a spike several mm. in length. Microscopically, they retained most of the characteristics of tongue epithelium, but the epithelial thickness was reduced in places. The dermo-epithelial junction was conspicuously papillated and the deeper layers of the epithelium were intensely basophilic. Rudimentary filiform papillary cores were often present, embedded in the dense horny overlay (Fig. 7).

The susceptibility of grafts of this type was studied in 13 guinea-pigs infected 9–148 days after grafting. All developed well marked macroscopic tongue lesions. With one exception, the susceptibility of the grafted epithelium was greatly reduced. Thus, in five, no lesions were found in the grafts, while in the remaining eight, necrotic foci (1–8 per graft) were present. They were usually $< 0{\cdot}2$ mm. in diameter, but one animal had lesions up to 1·5 mm. These foci consisted of small areas of epithelial cell necrosis accompanied by polymorph infiltration and sometimes evidence of local fluid formation. They were typical of FMD lesions in other situations.

Grafts of preputial epithelium on the chest

Preputial grafts consisted of smooth hairless areas up to 8 mm. in diameter, showing no tendency to accumulate keratin. Microscopically, the epithelium was fairly thick with a basal layer that tended to be more basophilic than that of the surrounding hairy skin (Fig. 8). Some of the large sebaceous glands of the prepuce were usually represented in the grafts.

Nine animals were infected 50–88 days after grafting and in most of them well marked secondary lesions developed in the prepuce. The susceptibility of the preputial grafts however was greatly reduced. In five of these animals, occasional minute foci ($< 0{\cdot}1$ mm. diameter) of epithelial necrosis with some polymorph infiltration were found in the grafts 2–3 days after infection. Usually, there were only 1–2 per graft but in one instance there were ten.

Grafts of metatarsal pad skin on the chest

Grafts of pad skin formed sharply defined hairless areas overlaid by dense keratin. Microscopically, they were clearly demarcated by their thick Malpighian layer, papillated dermo-epidermal junction and thick laminated stratum corneum (Fig. 9).

Seven animals bearing grafts of this type were infected intra-peritoneally 35–227 days after grafting. Macroscopic lesions involving at least about half the total metatarsal pad area developed 3–4 days after infection and the animals were killed after a further 1–5 days. No lesions were found however in the grafts.

After intradermal infection, three out of five guinea-pigs grafted for 46–174 days, had occasional minute foci (1–6 per graft ; $< 0{\cdot}15$ mm. diameter) of epidermal necrosis and polymorph infiltration when killed 2–5 days later.

Metatarsal pad skin grafted to the chest clearly undergoes a considerable reduction in susceptibility.

The lesion distribution in a deformed guinea-pig

The lesion distribution was studied in a guinea-pig with congenitally deformed fore limbs due to a carpal flexure. The animal's weight was born on the anterior carpal surface, the skin of which had become hairless, while the pads of the feet were out of contact with the ground. After infection widespread severe necrosis and some vesiculation developed in the carpal skin ; lesions in the pads, however, were limited to occasional microscopic foci.

Experiments on the nature of graft susceptibility

The change in susceptibility manifested by the grafts in their new situations provokes the question as to the nature of the tissue changes responsible. Some of the possible factors which it was thought might be concerned were therefore explored.

The role of epithelial mitotic activity.—The possibility was investigated that epithelial susceptibility might be dependent upon a high level of mitotic activity and that alterations in mitotic rate might explain the change in susceptibility produced by grafting. The relative mitotic activities of the grafts and their parent epithelia were compared in the same animals, at an environmental temperature of 21°. The results are shown in Table II.

Neither in different regions of the integument nor in the grafts was there any consistent correlation between the susceptibility of an epithelium and its mitotic activity. Hairy skin grafted to either the pad or the tongue had an increased mitotic activity whose extent however did not parallel the enhancement of susceptibility to the virus, since mitotic activity was greater in grafts on the tongue, yet grafts on the pad appeared to have the higher susceptibility. Grafting to the chest reduced the mitotic activity of tongue epithelium, but had no effect on prepuce grafts, while grafted pad epithelium showed a slight but significant increase. In all three, however, the susceptibility to the virus was reduced by grafting.

EXPLANATION OF PLATES

FIG. 1.—Graft of black hairy skin on the tongue, 34 days after transplantation.

FIG. 2.—An encysted graft of hairy skin within the tongue. Haematoxylin and eosin, × 10.

FIG. 3.—Severely necrotic tongue epithelium (above) and the undamaged wall of an encysted hairy skin graft (below) 2 days after infection. Haematoxylin and eosin. × 120.

FIG. 4.—Severe necrosis of a graft of hairy skin transplanted to the tongue 35 days previously. Graft boundaries are indicated by arrows. 1 day after infection. Haematoxylin and eosin. × 27.

FIG. 5.—Graft of pigmented hairy skin on the metatarsal pad 31 days after transplantation. Pigment spread is just commencing.

FIG. 6.—Severe necrosis of a hairy skin graft on the metatarsal pad 2 days after infection. Haematoxylin and eosin. × 66.

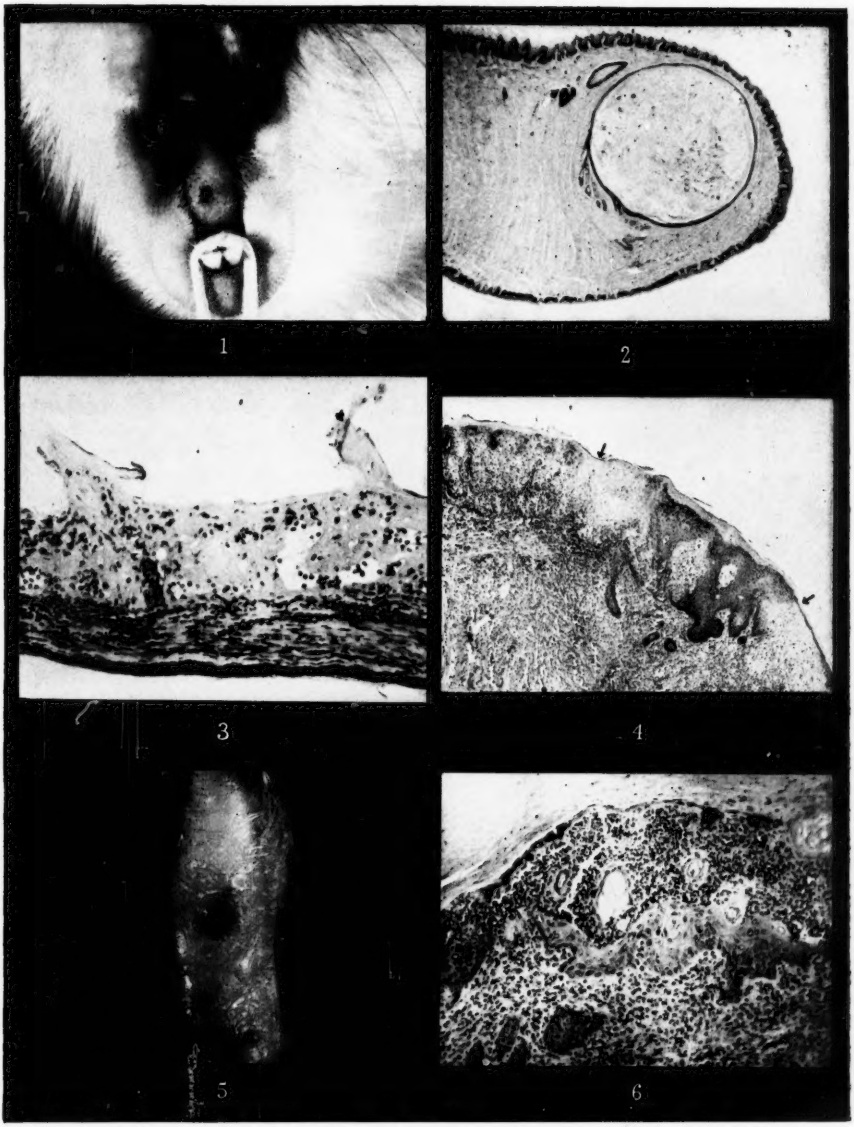
FIG. 7.—Tongue epithelium 87 days after transplantation to the chest. Haematoxylin and eosin. × 25.

FIG. 8.—Preputial epithelium 59 days after transplantation to the chest. Haematoxylin and eosin. × 14.

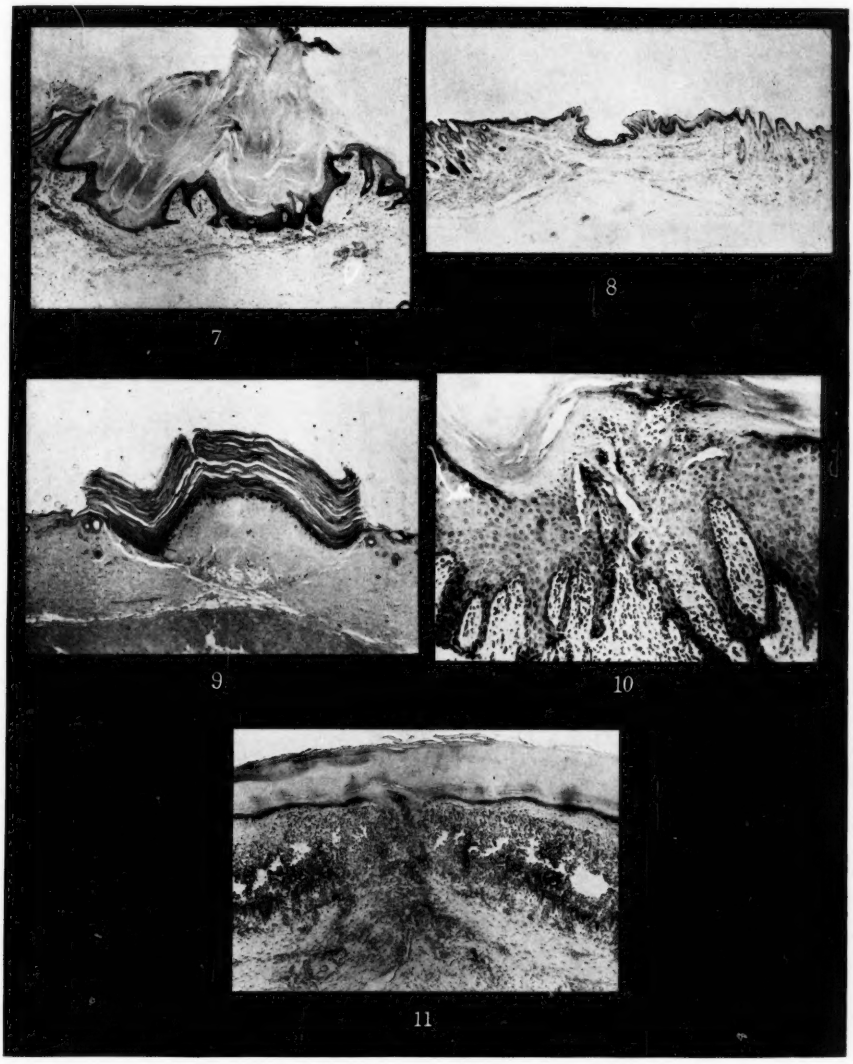
FIG. 9.—Metatarsal pad skin 227 days after transplantation to the chest. Haematoxylin and eosin. × 8.

FIG. 10.—A metatarsal pad graft on the chest 1 day after direct inoculation of virus. Lesions are limited to the immediate vicinity of the needle track. Haematoxylin and eosin. × 74.

FIG. 11.—Metatarsal pad 1 day after direct inoculation of virus showing part of the extensive area of necrosis around the needle track. The animal was the same as in Fig. 10 and the inoculation was performed similarly. Haematoxylin and eosin. × 25



Platt.



7

8

9

10

11

TABLE II.—*The Mitotic Activities of Paired Samples of Grafted and Ungrafted Epithelia and the Effect of Grafting on Epithelial Susceptibility*

Type of epithelium	No. of animals	Days grafted	Mean No. of mitoses per mm. of basal layer per 6 hr.	Mean differences between pairs and standard errors	P	Effect of grafting on susceptibility to FMD virus
Hairy skin	5	104–168	1·57	7·65±0·79	<0·001	Increased
Hairy skin→tongue			9·22			
Hairy skin	5	50–162	2·05	1·89±0·70	<0·10	,,
Hairy skin→met. pad			3·94			
Tongue	5	89–177	33·39	24·52±1·84	<0·001	Reduced
Tongue→chest			8·87			
Prepuce	5	107–125	5·92	0·43±1·38	<0·5	,,
Prepuce→chest			5.49			
Metatarsal pad	5	115–301	0·86	0·74±0·24	<0·05	,,
Metatarsal pad→chest			1·60			

Capillary permeability.—Owing to local traumatic influences, capillary permeability in the normal predilection sites is likely to be higher than on the chest, and this suggests a possible explanation for the loss of susceptibility on grafting to that site. Metatarsal pad grafts on the chest were therefore treated with histamine or *Cl. oedematiens* exotoxin (Elder and Miles, 1957) in order to render their capillaries permeable to circulating virus. The results are shown in Table III.

TABLE III.—*Effect of Local Increase in Capillary Permeability on Susceptibility of Metatarsal Pad Grafts to FMD Virus*

Treatment	No. of experiments	No. of focal microscopic lesions in the grafts (per 20 serial sections)	Extent of macroscopic generalisation to the tongue, lips, feet and prepuce*
Injection of histamine I.D. near graft margin : (1 × 0·80 ; 4 × 2·50 ; 1 × 11·10 μg histamine base)	6	3, 10, 10, 1, 5, 1	+ + to + + +
Histamine iontophoresis of the graft area	3	2, 6, 5	+ to + + +
Injectionof Cl. oedematiens toxin I.D. near graft margin (1/16,000 ; 1/10,000)	2	1, 1	+ + to + + +
Injection of saline I.D. near graft margin	4	0, 1, 8, 20	+ + to + + +

* Extent of generalization : + lesions at 1 site (tongue, lips, forefeet or prepuce)
+ + ,, ,, 2 sites
+ + + ,, ,, 3 or more sites

There was no evidence that either agent had any significant effect in rendering the grafts more susceptible. Small numbers of minute lesions, of the character and size already described, were found in many of the grafts but they occurred also in the saline injected control grafts.

Although concurrent infection with FMD virus may have reduced the sensitivity of the capillaries to histamine, their reactivity was not abolished since histamine iontophoresis gave rise to local vasodilatation and, in two instances, slight whealing. Moreover, intra-dermal injection of histamine (2·5 μg. base) into the flanks of 2 infected guinea-pigs, in the presence of circulating Evans Blue, was followed by prompt exudation of the dye at the histamine-treated sites. The guinea-pigs had been infected 1 day and showed severe primary lesions.

Effect of trauma on metatarsal pad grafts.—The effect of irritation on the susceptibility of grafted skin was investigated by treating the grafts and their neighbourhood with a mixture of turpentine and acetone (Friedewald, 1942). In experiments with uninfected animals, repeated treatment with this mixture produced necrosis of hairy skin but the grafts remained undamaged ; presumably they derived some protection from the thick stratum corneum.

Seven infected animals were treated with the mixture, 2 on the day of infection only and 5 on the following day as well. They were killed 2 days after infection. All the grafts contained small numbers of microscopical foci but in 2 animals, each of which had received 2 treatments, there were apparently specific marginal lesions accompanied in 1 case by moderately severe patchy necrotic lesions in the graft interior.

TABLE IV.—*Comparison of the Virus Content of Untreated and Irritated Metatarsal Pad Grafts*

	Experiment No.	Days grafted	No. of treatments	Virus content (Log. ID50/0·03 g. tissue)		Extent of generalization (macroscopic)
				Graft	Connective tissue	
Untreated grafts	1	18	0	2·8	2·2	Tongue, lips
	2	88	0	0	<1·5	Forefeet
	3	95	0	0	0	None
	4	102	0	1·6	≤1·5	Tongue, forefeet
	5	120	0	2·0	≤1·6	Tongue, lips, perineum
After painting with Turpentine/Acetone mixture	6	32	1	0	0	Lips
	7	42	2	3·3	2·3	Tongue, lips, prepuce
	8	88	2	3·8	3·6	Tongue, lips, prepuce
	9	121	2	>5·7	3·7	Tongue, lips
	10	123	2	4·9	3·7	Tongue, lips, perineum

The results of titrations of the virus content of irritant-treated and untreated grafts are shown in Table IV. In blood-free, untreated grafts, virus was either absent altogether or present in a low titre not significantly higher than for the underlying connective tissue where the virus is presumably present in the intercellular fluid. In irritant treated grafts, however, the virus titres in 3 instances were significantly higher than in the connective tissue. These results suggest that little or no virus multiplication occurs in untraumatized metatarsal pad grafts but when the graft is irritated, a slight increase in susceptibility to the virus takes place. The increased virus content of the connective tissue in the irritant treated animals in these experiments was probably due to local capillary permeability and leakage of virus into the intercellular fluid where it is inaccessible to perfusion.

Susceptibility of metatarsal pad grafts to direct inoculation.—Five grafts, 43–56 days after transplantation, were inoculated directly *via* single needle tracks flooded with virus. In each animal, a similar injection was made into one metatarsal pad. The animals were killed 1 day later. All the injected metatarsal pads showed severe necrosis extending out for about 2 mm. on each side of the track. One of the grafts showed lesions which were only slightly less severe than in the metatarsal pad. The other four grafts however displayed only minimal involvement, with local epithelial degeneration and mild polymorph infiltration in the immediate neighbourhood of the track. These lesions showed no tendency to extend into the surrounding graft epithelium and they were probably due partly to trauma produced by the needle (Figs. 9 and 10).

DISCUSSION

The pattern of lesion distribution observed in foot-and-mouth disease is also a feature of several unrelated dermotropic virus infections of animals. Thus, the viruses of vesicular stomatitis, vesicular exanthema, contagious pustular dermatitis and mucosal disease all show an affinity for the hairless parts of the skin and for the buccal epithelium in their respective hosts. In foot-and-mouth disease, the distribution pattern of epithelial lesions is maintained throughout a wide range of host species, including birds (Skinner, 1954) and relatively hairless species such as the pig. Epithelial lesions may be absent, however, in very young animals where the virus tends to be myotropic and produces myocardial and skeletal muscle damage.

In man, fully authenticated instances of foot-and-mouth disease are exceedingly rare but in a number of cases, where the diagnosis appears to have been established lesions were found in the mouth, hands, feet and nail beds and were similar in type to those observed in other species (Platt, 1958*b*). In children, an epidemic characterised by aseptic meningitis, herpangina and vesicular lesions on the fingers has been reported, from which a Coxsackie A virus was recovered (Dalldorf, 1958).

The present investigations were undertaken with the object of attempting to shed some light on the factors responsible for the localization of lesions in foot-and-mouth disease. All the predilection sites are areas exposed to irritation or trauma of a normal physiological character, and grafting epithelium from these sites to a non-traumatized environment such as the side of the chest leads to a considerable loss in its susceptibility. Conversely, hairy skin from the flank grafted to the tongue dorsum or the metatarsal pad becomes distinctly susceptible, more so in the latter site than in the former. Thus, there appears to be a correlation between epithelial susceptibility and exposure to persistent trauma, and the development of susceptibility in the carpal skin of a deformed guinea-pig indicates that this susceptibility may be manifested in locations other than the usual predilection sites. These results extend and confirm the findings of Maitland (1928) although the techniques used are different.

In general, the epithelia of the predilection sites are of a hyperplastic character. Their histological characteristics and differentiation are well retained after grafting although their susceptibility is not. Evidently, persistence of an irritant stimulus is required for the retention of susceptibility. On the other hand when hairy skin is grafted to the tongue or the pad, while it retains many of its

characteristics, it also undergoes a pronounced epidermal hyperplasia at the same time as it becomes susceptible. A similar connection between an actively hyperplastic state of the epithelium and susceptibility to the virus has already been reported in the case of the teats of female guinea-pigs (Platt, 1958*a*). These are normally not a predilection site but with the hormonal stimulation of pregnancy they become hyperplastic and, coincident with the onset of lactation, susceptible to the virus.

The present experiments suggest that localization in the predilection sites cannot be explained solely in terms of either local capilliary permeability, as was suggested by Burrows (1932), epithelial mitotic activity, or (from unpublished data) regional temperature pattern. While increased capilliary permeability may well be an accessory agency in the localization of lesions at the predilection sites, it is unlikely that it is the only factor since increasing the permeability of the local capillaries failed to restore susceptibility to pad skin that had been grafted to the chest.

It seems most likely that the reduced susceptibility to secondary lesions displayed by the epithelia grafted to the chest is attributable to a loss of susceptibility of the constituent cells themselves, since in 4 out of 5 instances, even direct inoculation of virus into pad grafts on the chest gave rise to only minimal, non-progressive lesions. The nature of the cellular properties on which susceptibility to FMD virus depends is at present completely obscure and further investigations on this problem are in progress. Secondary lesions in grafted pad skin were confined to occasional minute self-limiting lesions and little or no virus was present in the graft. Presumably the virus is unable to penetrate the cells, or having penetrated is unable to multiply. Acute chemical irritation of such grafts however, produced rather irregularly, a slight increase in susceptibility.

It seems possible that persistent irritation leads to metabolic or cell-surface modifications in the cells which may favour virus infection. Biochemical data on the metabolic activity of the epithelial cells at the predilection sites, compared with the rest of the skin, would be of considerable interest. It is significant that most of the predilection sites are fairly vascular and in the case of the feet, alterations in blood flow have an important bearing on the development of lesions in the pads. Guinea-pigs kept at a somewhat higher temperature than is customary have more susceptible pads and this is seen particularly well with unadapted strains of virus at high dilutions. For this reason, infected guinea-pigs in this Institute are kept at 21°. Conversely, susceptibility is much impaired by peripheral cooling and vasoconstriction arising from a chilly environment. The reduction of pad susceptibility in guinea-pigs suspended in nets (Lotze, 1934) is probably also attributable to circulatory factors, since Schach (1935) found that the feet became cold due to venous stasis ; by keeping the suspended animals at 37° blood flow through the feet was improved and susceptibility retained. Venous stasis due to restriction of limb movement by bandaging may play a part in reducing the susceptibility of the metatarsal pad in the experiment described by Maitland (1928). In an extremity such as the foot, skin temperature, blood flow and tissue metabolic activity constitute a complex of mutually inter-related factors, and suboptimal temperature conditions or reduced blood flow are probably accompanied by a depression of local metabolic activity in the metatarsal pad epithelium which would counteract any advantage which the site otherwise affords for virus infection.

It is possible that the differences in susceptibility between the predilection sites and hairy skin may be relative rather than absolute. It has been noted that although hairy skin does not develop secondary lesions, differences exist between the skin in different regions in response to direct inoculation of virus. The skin of the legs when thus tested was more susceptible than the skin of the back (Platt, 1958*a*). It is also known that cattle infected with very virulent strains of FMD virus may occasionally display lesions in the hairy skin of the thighs. These observations would suggest that whatever the factors may be which determine epidermal susceptibility, they may be present in quantitatively lesser degrees in sites other than those normally affected by the virus.

SUMMARY

It has been shown that after grafting to the tongue or the metatarsal pad, hairy skin becomes susceptible to the virus of foot-and-mouth disease.

The susceptibility of epithelium from the predilection sites (tongue, prepuce and metatarsal pad) to infection with the virus is greatly reduced after grafting to the chest.

There is no close relationship between the susceptibility of a graft and its mitotic activity and it has not been possible to restore the susceptibility of pad grafts on the chest by increasing local capillary permeability.

Application of an irritant to pad grafts on the chest gave rise to a slight, but irregular, increase in graft susceptibility.

Four out of five pad grafts were relatively insusceptible to direct inoculation of virus, which suggests that the loss of susceptibility which such grafts undergo involves a change in the cells themselves.

It was concluded that susceptibility is related to a hyperplastic state of the epithelium and persistent local irritation and it is suggested that in regions where these conditions are present, the metabolic activities and cell surface properties of the epithelial cells may differ from elsewhere in the skin and in a way favorable to virus infection.

I should like to thank Mr. J. R. P. Dale and Mr. R. R. H. Hill for their valuable assistance during the course of this work. I am indebted to Mr. A. Thomson of the Wellcome Research Laboratories for providing me with the sample of *Cl. oedematiens* toxin.

REFERENCES

Billingham, R. E. and Medawar, P. B.—(1951) *J. exp. Biol.*, **28**, 385.

Burrows, H.—(1932) " The Localization of Disease '. London (Baillière, Tindall and Cox).

Dalldorf, G.—(1958) *Mod. Med., Lond.*, **3**, 1051.

Elder, J. M. and Miles, A. A.—(1957) *J. Path. Bact.*, **74**, 133.

Friedewald, W. F.—(1942) *J. exp. Med.*, **75**, 197.

Lotze, H.—(1934) *Z. Hyg. InfektKr.*, **116**, 556.

Maitland, M. C.—(1928) *J. comp. Path.*, **41**, 150.

Platt, H.—(1958*a*) *J. Path. Bact.*, **76**, 119.—(1958*b*) *Med. Pr.*, **240**, 1195.

Schach, W.—(1935) *Z. Hyg. InfektKr.*, **117**, 149.

Skinner, H. H.—(1954) *Nature. Lond.*, **174**, 1052.

TWO-PHASE ULCERATION IN GUINEA-PIG SKIN FOLLOWING LOCAL IRRADIATION WITH X-RAYS

P. G. MELLETT, J. B. WALTER AND L. E. HOUGHTON

From Harefield Hospital, Middlesex and the Royal College of Surgeons of England, London, W.C.2

Received for publication September 5, 1959

IT has been shown that necrosis of small areas of guinea-pig skin due to localised X-irradiation may be modified and delayed for long periods by treatment with parenteral cortisone (Houghton, Walter and Jones, 1954). To attempt to understand the mechanism of this effect, it appeared necessary to know the microscopical appearances associated with naked-eye changes in the irradiated areas. Though the microscopical appearances of irradiated guinea-pig skin had been described (Frédéric, 1949), no adequate account was available of such appearances over a long enough period following heavy dosage of X-irradiation. An appropriate investigation was therefore undertaken.

MATERIALS AND METHODS

Twenty-two young adult albino guinea-pigs (average weight 705 g.) were irradiated over a circular field (2·6 cm. diameter) on both flanks. Their backs had been previously clipped over the areas to be used. Care was taken to avoid other injuries to the area to be irradiated. Each animal received an exposure dose of approximately 6000 r. in a single irradiation of 3 minutes' duration to each field. Before irradiation the animals were anaesthetized with Nembutal (2 mg. per 100 g. body weight) given by intraperitoneal injection.

Life-size drawings were made of the 2 irradiated areas on each of the guinea-pigs at 4 day intervals. Immediately following drawing, the irradiated area was excised completely from one side of one previously untouched animal. Ether anaesthesia was used for the excision.

The excised tissue was fixed in 10 per cent formol saline, paraffin sections were prepared in the usual way and stained with haematoxylin and eosin, Van Gieson's stain, and periodic acid Schiff (P.A.S.).

It was noticed that, in this and other experiments, when 2 areas were similarly irradiated in any one animal on the same day, much the same naked eye appearances occurred in both areas at any subsequent observation time. Developments in the irradiated area of skin left intact were therefore held to give a good idea of what would have happened to the excised area had it been left untouched. It was therefore possible to assemble the various biopsy specimens into an order representative of the correct series of changes from irradiation to ulceration and subsequent healing.

It was noted also that there was a variation of one to several days in the timing of any specific stage in the development of ulceration from one guinea-pig to another. The range of time over which a particular stage in a series of post-irradiation changes might be seen is mentioned with the description of each stage.

Irradiation technique

The X-ray beam was generated at 60 kV., 2 mA (constant potential) the h.v.t. being $0{\cdot}5_5$ mm. Al. using a Philips' contact therapy apparatus. The circular irradiated field was defined by a short closed-ended Perspex applicator 2·6 cm. in diameter (Fig. 1). The short focus-skin distance, 3·0 cm., ensured a rapid decline of dose beneath the skin; axial depth doses measured in a water-equivalent medium ("Mix D", Jones and Raine, 1949) showed

that the dose fell to 50 per cent of the surface value at 3·2 mm., and to 10 per cent at 16 mm. beneath the surface. The surface dose rate was measured in air by means of a shallow cylindrical ionisation chamber 1·5 mm. deep made of graphite. Using an extrapolation method, the dose rate on the emergent surface was found to be 2010 r. per min. Backscatter from the tissues would increase this dose by about 5 per cent (Oosterkamp, 1950). Photographic film exposed in contact with the applicator face showed that the field was uniformly irradiated and was sharply confined to the prescribed circular area.

The exposure dose rate of the superficial skin was therefore taken as 2060 r. per min. (to an estimated accuracy of 5 per cent). This would correspond to an absorbed surface dose in 3 min. exposure of about 5750 rad in soft tissue (N.B.S., 1956).

RESULTS

Phase I

A. Changes leading to superficial ulceration

(*i*) *Macroscopic.*—The irradiated area could not be distinguished from the surrounding non-irradiated area for about 10 days. Between the 11th and 18th days after irradiation hair-growth was noticed to have ceased. It was thought probable that this had occurred a day or two before it was clinically apparent. A faint erythema of the skin appeared at the same time as hair-growth was seen to have ceased. From the 14th day hair was shed—sometimes diffusely and sometimes in patches. Epilation was usually complete by the 20th day and invariably by the 32nd day. Desquamation was noticed often, both in association with patchy hair loss and over already epilated areas.

(*ii*) *Microscopic : The epithelial changes.*—(*a*) At 4 days the epithelium was slightly thickened, the cells showing enlargement of the nuclei and some clumping of the chromatin (Fig. 2 and 3). Degenerative changes such as karyorrhexis and karyolysis were seen to be well marked on the 12th day. At this time the cytoplasm of the epithelial cells was more abundant and the general arrangement of cells was considerably distorted and hyperkeratosis was seen. One of the most striking features was enlargement of the nucleoli which was first detected by the 4th day and was well marked in the 12th day and subsequent specimens (Fig. 3–7). A few micronuclei were present at 4 days and these became progressively more abundant (Fig. 4). The nuclei became more pleomorphic and from the 12th day onwards multinucleated masses could be found (Fig. 6). Abnormal mitoses and characteristic pairing of nuclei were also noted (Fig. 5).

In the area where irradiated skin had joined the normal, the epithelium behaved in a different way, showing hyperplasia of the basal cells with many mitoses. The changes were seen by the 12th day, and resulted in the formation of a ring of thickened epithelium composed of cells which looked much more normal than those at the centre. This hyperplasia continued and became most marked at a distance of about 3 mm. inwards from the junction of irradiated skin with normal skin.

(*b*) *The hair follicles.*—The neck of the follicles behaved in the same way as the adjacent epithelium. By the 8th day the activity of the cells of the germinal matrix had fallen off and these structures subsequently disappeared. The root sheathes underwent steady atrophy and were small by the 22nd day.

(c) *The connective tissues.*—By the 8th day the fibroblasts became somewhat enlarged and in the subepithelial layer occasional lymphocytes and polymorphs were present. These changes slowly increased and by the 22nd day fibroblasts

of the subcutaneous layer were enlarged, the changes being more marked than in the sub-epithelial layer. The blood vessels showed no detectable change.

B. Superficial ulceration

(*i*) *Macroscopic.*—From the 18th day in many animals, and in a few animals up to the 55th day, epithelium was lost. This was for a period of a few days only and usually over one or two small patches in the more central part of the irradiated area. It never occurred at the periphery.

This epithelial loss gave rise to wet, superficial ulcers (" excoriations ") which dried within 24 hr. and sometimes healed within a few days. Usually healing did not occur but the superficial ulcer appeared to progress directly to a deep ulcer (Phase II).

In some animals no superficial ulceration occurred though they were later to go on to deep ulceration.

(*ii*) *Microscopic.*—In the centre of the irradiated zone the epithelial changes progressed and were marked by the 22nd day ; areas of thinning appeared (Fig .7) and subsequently much of this abnormal epithelium underwent necrosis.

The loss of epithelium resulted in a shallow ulcer with much polymorph infiltration in the base (Fig. 12). The deeper connective tissues showed infiltration with inflammatory cells and considerable increase in the number of large, bizarre fibroblasts. Some of these cells could be seen in mitosis.

The hyperplasia of the outer ring of epithelium continued and the involved hair follicle sheaths formed keratin-filled cysts (Fig. 8). These were largest at a point about half-way between the centre and the circumference of the irradiated area. These cysts, together with the overlying hyperplasia of the epithelium, accounted for the raised rings seen later naked-eye (Fig. 13).

EXPLANATION OF PLATES

FIG. 1.—Animal in position for irradiation, showing the X-ray tube with special perspex applicator.

FIG. 2.—Normal guinea-pig skin. H. and E. ×410.

FIG. 3.—Four days after irradiation. The epithelial cells are enlarged. H. and E. ×410.

FIG. 4.—Twelve days. Further enlargement of cells. The nucleoli are very large and two cells with micronuclei are present (shown by arrows). H. and E. ×410.

FIG. 5.—Sixteen days. Epithelial cells are irregular and nucleoli very obvious. A pair of nuclei is seen on the left and there is abnormal mitosis. H. and E. ×410.

FIG. 6.—Sixteen days. Bunches of nuclei are seen. H. and E. ×410.

FIG. 7.—Twenty days. This irregular epithelium will shortly ulcerate. One micronucleus is shown. H. and E. ×410.

FIG. 8.—Twenty-eight days. Section through rampart showing thickened epithelium and underlying keratin filled cysts. H. and E. ×140.

FIG. 9.—Twenty-eight days. Epithelial hyperplasia in centre of the lesion preceding deep ulceration. This animal never showed superficial ulceration. H. and E. ×140.

FIG. 10.—Thirty-four days. Arteriole showing fibrinoid necrosis. H. and E. ×350.

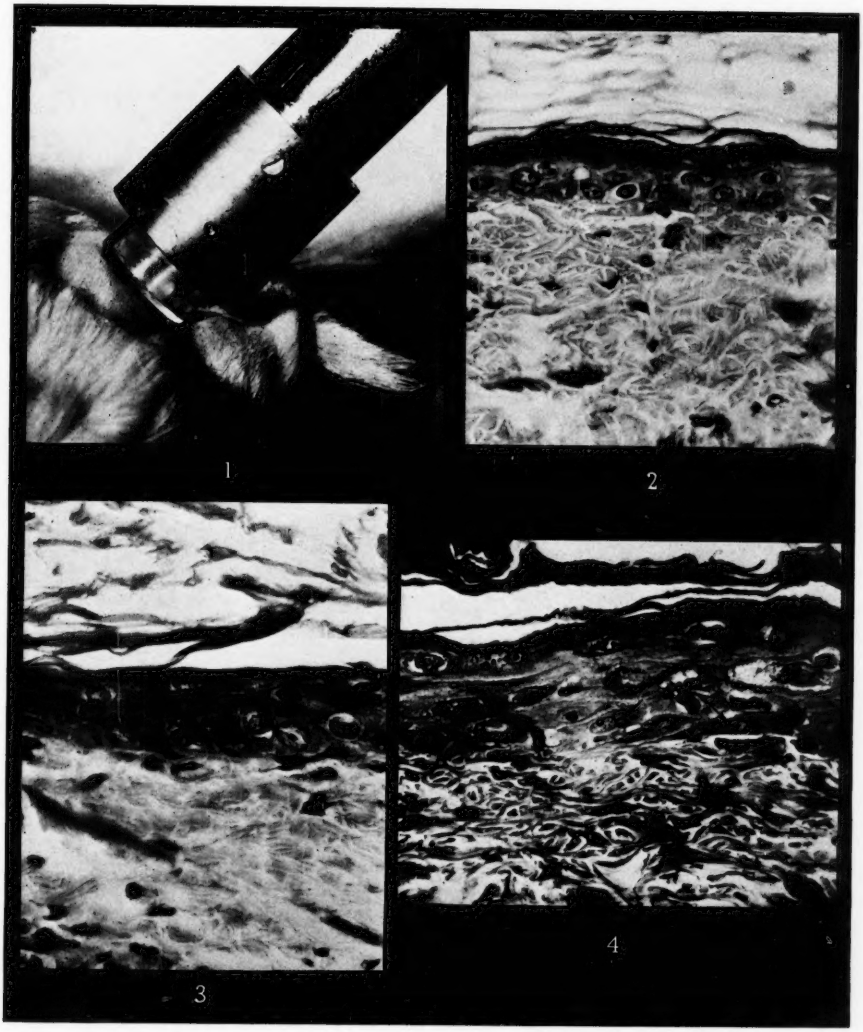
FIG. 11.—Seventy-four days. Hyperplastic epithelium covering the healed deep ulcer. H. and E. ×140.

FIG. 16.—Irradiated skin showing rampart formation.

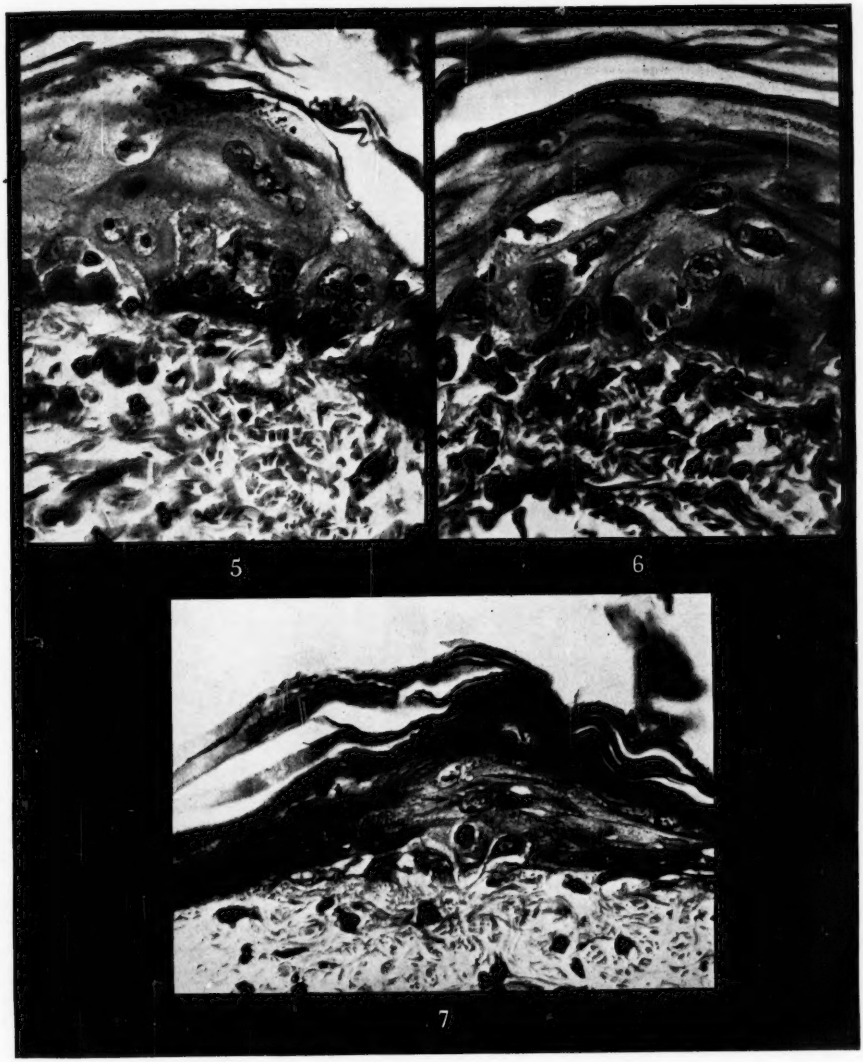
FIG. 17.—Deep ulceration commencing in the ramparts.

FIG. 18.—Fully developed ulcer. The irradiated area has contracted.

FIG. 19.—Healing deep ulcer.



Mellett, Walter and Houghton.

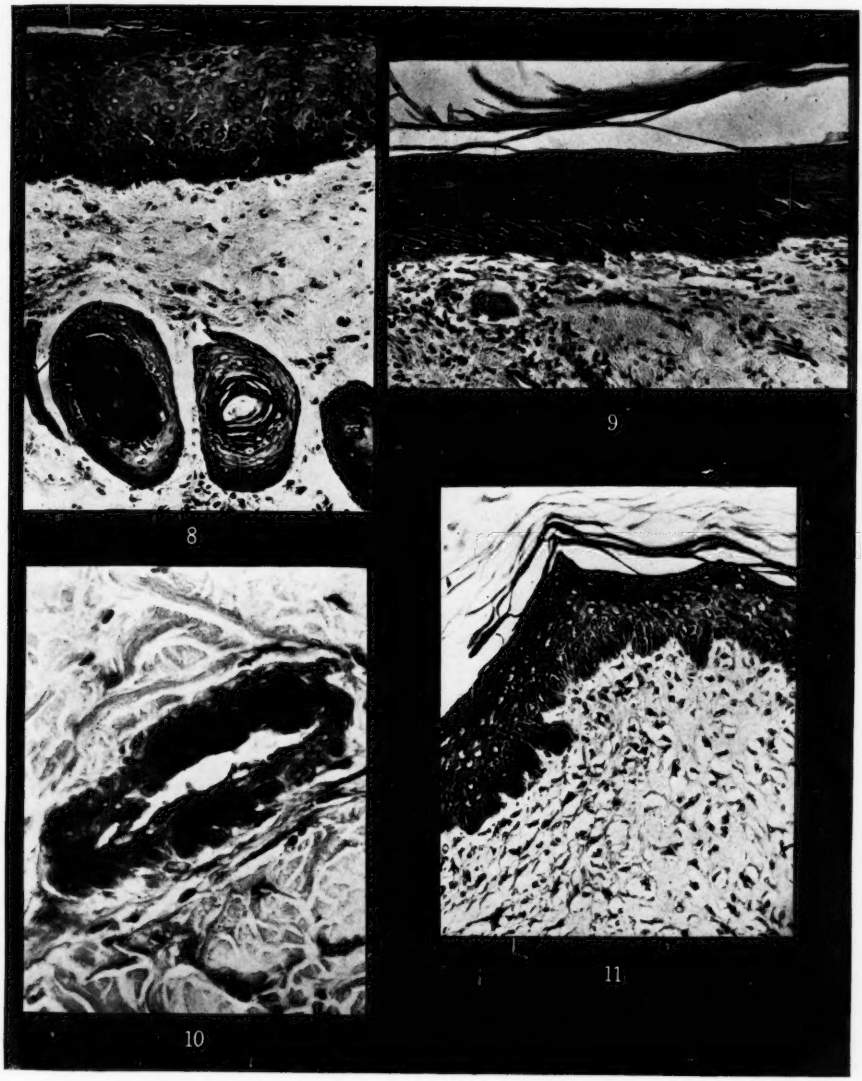


5

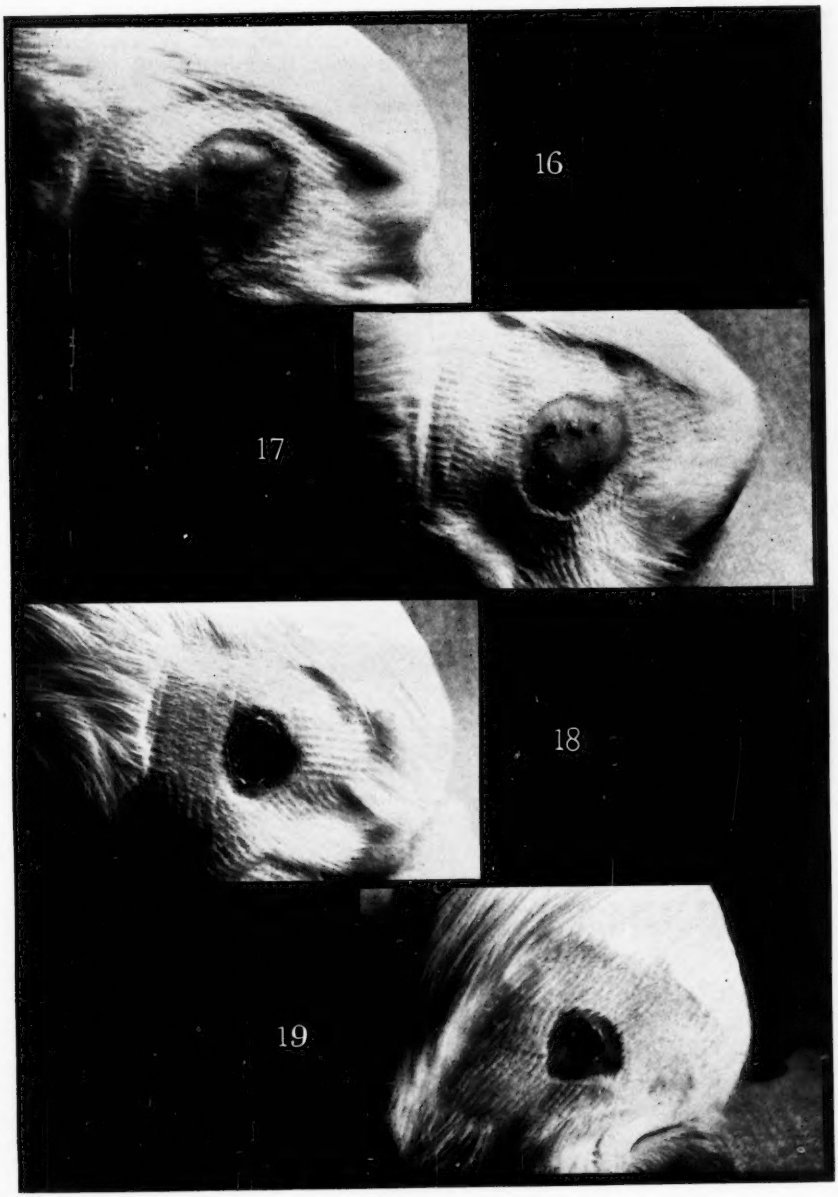
6

7

Mellett, Walter and Houghton.



Mellett, Walter and Houghton.



Mellett, Walter and Houghton.

In all cases the epithelium attempted to grow over the ulcerated area and in some cases this resulted in healing. In a few cases superficial ulceration did not occur and in one of these cases the whole area was covered by hyperplastic epithelium (Fig. 9). Occasional micronuclei were present in this epithelium.

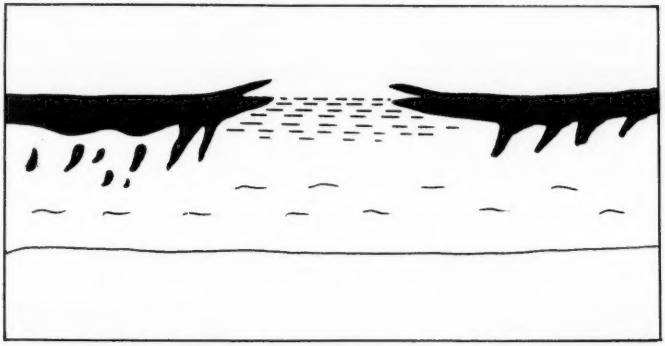


FIG. 12.—Superficial ulceration. This figure and Fig. 13, 14 and 15 show the phases of ulceration following irradiation. These diagrams are not to scale, the thickness of the epithelium is exaggerated.

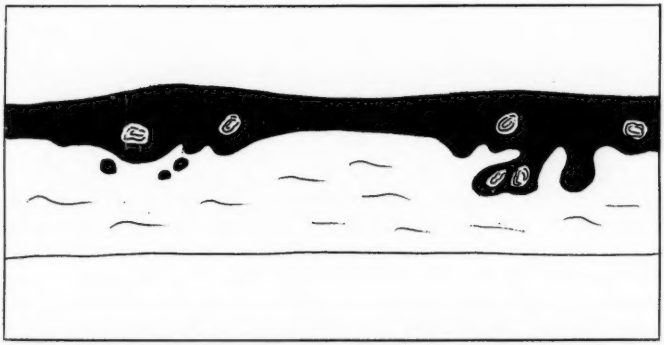


FIG. 13.—Superficial ulcer healed and raised " ramparts " formed by gross hyperplasia of epidermis and hair follicles half-way between the centre and the margins of the irradiated area.

Phase II

A. *Deep ulceration*

(*i*) *Macroscopic.*—In the majority of animals superficial ulceration progressed directly to deep ulceration between the 26th and the 48th post-irradiation day. The small weeping area dried and a thin scab formed. Meanwhile a larger circular area which included the superficial ulcer was noticed to become brownish then blackened. The result was the formation of a large deep ulcer (Fig. 18). However, in a few cases the same type of ulcer was formed after the following more complicated series of changes. The superficial ulcer healed and was followed

by an apparently quiescent interval lasting for a few days between the 20th and 40th post-irradiation days. The epilated skin was white, usually smooth and often showed a few tiny flakes of desquamated epithelium. Between the 25th and 50th days a faintly pink, often incomplete, slightly raised rampart-like circle then appeared about half-way between the centre and the circumference of the irradiated area (Fig. 13 and 16). It was about 3–4 mm. wide and 1 mm. deep. There were occasional bulges in the contour of these " rampart " rings. There were often patches of dry flaking epithelium on their surface. Segments of the ramparts gradually turned brownish (the colour appearing to be under the surface). These segments gradually darkened until they became blackish and necrotic in appearance (25th to 50th day) (Fig. 17). Simultaneously the centre of the irradiated area became blackened and between the 27th and 60th days a button-like

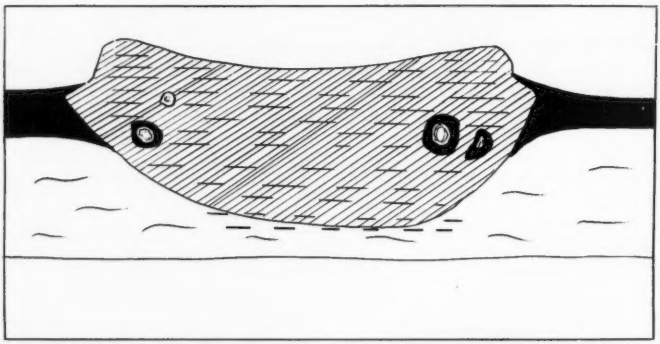


FIG. 14.—Deep ulceration involving hyperplastic epidermis including region of rampart formation and underlying connective tissue.

mass of dry necrotic skin was produced surrounded by indurated and sometimes erythematous skin. The whole irradiated area showed marked shrinkage (Fig. 18).

(*ii*) *Microscopic.*—From the 26th to the 48th day changes occurred in the dermis coinciding with deep ulceration. The connective tissue of the dermis, the subcutaneous tissue, and later the muscle, underwent necrosis. This was associated with blood vessel changes. These consisted of swelling of the endothelial cells and the intimal connective tissue, fibrinoid necrosis of the whole wall (Fig. 10), and in some cases actual thrombosis. Areas of haemorrhage were also seen. The epithelium underwent necrosis and a large deep ulcer was formed (Fig. 14). Although there was some polymorph infiltration adjacent to the ulcer, the most salient features of the surrounding connective tissue response were the presence of bizarre fibroblasts and PAS-positive material between the cells.

B. *The healing stage*

(*i*) *Macroscopic.*—After a few days (56th day onwards), the dry blackened necrotic central mass rose above the level of the surrounding skin, which undermined it, producing a roughly bevelled edge. The base of the necrotic epithelium was gradually narrowed until it was a shallow cone or mushroom-like structure

(Fig. 15 and 19), which was at last cast off (60th day onwards), leaving a small elliptical scar in an area of epilated skin much smaller than the original irradiated area. Subsequently this became shrunken still further until the final scar was difficult to find in the hair growing from the surrounding normal skin.

(*ii*) *Microscopic.*—The epithelium adjacent to the ulcer underwent tremendous hyperplasia sending in tongues of cells which insinuated themselves between the scab and the underlying living tissue. The scab was thus undermined and became mushroom-shaped. Finally it was cast off and the whole surface was epithelialized. The cells were large and had pale nuclei but by the 74th day a new basal layer was formed and was composed of more normal-looking cells (Fig. 11).

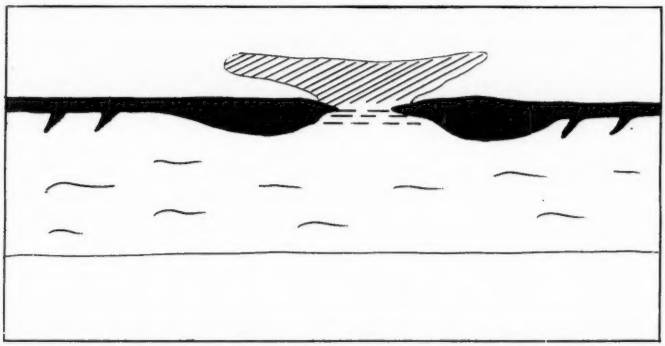


FIG. 15.—Healing of ulcer by ingrowth of epithelium undermining necrotic tissue.

In the deeper tissues the healing processes involved the formation of a granulation tissue containing many small, thin-walled capillaries and bizarre fibroblasts (Fig. 11). Collagen formation resulted in the formation of a scar and from the 65th day endarteritis obliterans could be seen in some small vessels.

DISCUSSION

The " superficial ulceration " described here occurring from the 22nd day clearly corresponds with the ulceration of guinea-pig skin described by Frédéric (1949). It also corresponds with the ulceration of mouse and rat skin described elsewhere. Argyris (1954) states that this occurs in mice at the 14th day where hair follicles are active and at the 16th to 23rd days where hair follicles are resting. (In the mouse hair growth is occurring in some areas while others are " resting ". This is not so in the guinea-pig where all the hairs of any given area are not in a growing or resting state at any given times (Dawson, 1930).)

Devik (1955) describes superficial necrosis with epithelial loss and exposure of the dermis in hairless mice on the 9th to 10th days after irradiation of an area of skin 10·5 × 9 mm. with 2700 r.

Jolles (1941) similarly describes superficial ulceration in the skin of the rat from the 8th to 26th day after irradiation with 2500 r. to an area 1·7 cm. in diameter and from the 12th to the 22nd day after irradiation with 3500 r. to an area of 3 cm.

Although hyperplasia of irradiated tissue is well known, its subsequent necrosis as a second phase of ulceration is not widely recognized. It must be emphasized that this second phase which we have observed is quite distinct from the ulceration of atrophic skin occurring as a late result of irradiation and often seen clinically.

Frédéric (1949) emphasises the great thickening of the epidermis 14 days after ulceration due to irradiation with 3000 r. Argyris, Devik and Jolles all describe a peripheral area of hyperplasia at the time of ulceration. Devik mentions that 3 out of 6 mice biopsied at the 13th–14th day showed hyperplastic epithelium over the whole irradiated field. This hyperplasia gradually receded and was gone by a little over 20 days from irradiation. Hyperplasia at the border of an irradiated field is also well known from clinical experience in radiotherapy. Miescher (1925) describes epithelial thickening as a regular feature following ulceration due to X-irradiation of an area of human skin 4×4 cm. Such hyperplasia is not necessarily dependent on a central area of epithelial loss, though hyperplasia and inward migration is the normal method of repairing an ulcer (Devik, 1955).

None of these authors has reported subsequent necrosis of the hyperplastic epithelium. However, we find that this occurs during the second phase of deep ulceration. In one of our animals necrosis occurred in the hyperplastic epithelium which covered the whole irradiated field, no superficial ulceration having taken place. In several animals, deep ulceration occurred after healing of initial superficial lesions, whilst in others the hyperplasia adjacent to the superficial ulcers was involved in the subsequent deep ulceration. It is of interest that in some animals the necrosis of hyperplastic epithelium was seen to begin where it was deepest—*i.e.* in the region of the " rampart " rings, where the hyperplastic hair follicles had formed keratin-filled cysts.

It will be noted that this later necrosis (" deep ulceration "), occurred concurrently with marked histologically observable damage in the dermis, including pathological changes in blood vessels. While the histological findings regarding the blood vessels of the dermis at the time of " superficial ulceration " support Devik's view that blood vessel changes play no part in the production of superficial epidermal necrosis, the findings at the time of the later necrosis of hyperplastic epithelium suggest that vascular changes may be the prime cause.

It is, however, possible that the necrosis of hyperplastic epidermis is due to some other factor. It has been suggested (Chase, personal communication) that this may be a lack of cohesion in cells greatly damaged by irradiation, and that these are cast off and replaced by less abnormal cells growing in from the periphery.

It may be that necrosis of the hyperplastic cells occurs owing to some disturbance of cellular metabolism associated with an excessive rate of mitosis. However, although guinea-pig skin shows marked hyperplasia following initial ulceration due to exposure to CO_2 snow, a 2nd phase of deep ulceration does not occur (Firket, 1951).

In view of the protective action of normal surrounding skin (Jolles, 1941), the size and shape of the irradiated area may play some part in this phenomenon.

Possibly a combination of increased demand for nutritional material by the hyperplastic cells and decreased blood supply due to blood vessel damage accounts for this second phase of ulceration.

Although it is not clear why guinea-pigs show these 2 phases of ulceration, the periodicity of post-irradiation changes has been noted previously. Thus Warren (1943) described 2 phases of erythema, one occurring in a few hours to 3 days of iradiation and a second phase 10 to 28 days later. Miescher (1925) described 3 phases of erythema, the maximum histological changes occurring during the 3rd phase in the 6th week. This periodicity in the development of post-irradiation changes and the occurrence of latent intervals is a highly, characteristic but as yet unexplained feature of radiation damage. It is, however, likely that different mechanisms are involved in the production of radiation damage during these different phases. In the study of the action of protective substances it is important that the phases should not be confused.

SUMMARY

Twenty-two adult albino guinea-pigs were exposed to an X-ray dose of approximately 6000 r. over a circular field 2·6 cm. in diameter on both flanks. One irradiated area of skin was excised from successive animals at intervals of 4 days. The remaining irradiated areas were untouched and their appearances were observed. Two phases of ulceration were observed: superficial ulceration occurred from the 18th day onwards and consisted of epithelial loss but little or no damage to underlying connective tissue. The changes preceding ulceration were largely restricted to epithelial cells; enlargement of cells and the formation of micronuclei and multinucleated masses was seen. Deep ulceration occurred in all animals and sometimes followed directly on superficial ulceration. In other animals the superficial ulcers healed and a circular raised "rampart" was formed midway between the centre and the edge of the irradiated area. Rampart formation was also seen in one animal which had shown no superficial ulceration.

The rampart was composed of hyperplastic epithelium further elevated by underlying hyperplastic remnants of hair follicles. Deep ulceration occurred first in this (rampart) zone of hyperplasia before involving the centre of the irradiated area. Microscopically there was necrosis of all layers of skin as well as underlying tissues including muscle. Blood vessels showed changes including thrombosis and fibrinoid necrosis. It is suggested that the cause of the early superficial ulcers differs from that of the later deep ulcers; it is noted that only this 2nd phase of deep ulceration is accompanied and possibly caused by vascular damage.

We wish to thank the British Empire Cancer Campaign for a grant to study radiation damage and the Cancer Research Committee of Mount Vernon Hospital for their co-operation. We are greatly indebted to Mr. D. E. A. Jones for his advice and assistance with X-ray technique. We thank Mr. A. L. E. Baron for help with the photography.

REFERENCES

ARGYRIS, T. S. R.—(1954) *Amer. J. Anat.*, **94**, 439.
DAWSON, H. L.—(1930) *Ibid.*, **45**, 461.
DEVIK, F.—(1955) *Acta radiol. Stockh., Suppl.*, 119.

FIRKET, H.—(1951) *Arch. biol., Paris*, **62**, 309.
FREDERIC, J.—(1949) *Ibid.*, **60**, 79.
HOUGHTON, L. E., WALTER, J. B. AND JONES, D. E. A.—(1954) *Brit. med. J.*, ii, 1313.
JOLLES, B.—(1941) *Brit. J. Radiol. N.S.*, **14**, 110.
JONES, D. E. A. AND RAINE, H. C.—(1949) *Ibid.*, **29**, 196.
MIESCHER, C.—(1925) *Arch. Derm. Syph., N.Y.*, **148**, 540.
NATIONAL BUREAU OF STANDARDS.—(1956) Handbook 62—(I.C.R.U. Report).
OOSTERKAMP, W. J.—(1950) *Acta radiol., Stockh.*, **33**, 491.
WARREN, S.—(1943) *Arch. Path. (Lab. Med.)*, **35**, 34.

THE INFLUENCE OF DIETARY DEPLETION OF THIAMINE, RIBOFLAVIN AND NIACIN ON THE INDUCTION OF GLIOMATA IN MICE

J. M. BRIDGES

From the Department of Pathology, Queen's University, Belfast

Received for publication September 18, 1959

THE first report of the successful production of gliomata by implantation of pellets of chemical carcinogens into the brains of experimental animals was that of Seligman and Shear (1939); they implanted 1·5 mg. pellets of methylcholanthrene in the right cerebral hemisphere of 20 C_3H mice and of these animals 11 subsequently developed gliomata. These results were confirmed by Zimmerman and Arnold (1941) who later showed that benzpyrene (Arnold and Zimmerman, 1943) and dibenzanthracene (Zimmerman and Arnold, 1943) also had a local carcinogenic action when implanted intracerebrally. These workers (Zimmerman and Arnold, 1944) also investigated the response of various strains of mice to intracerebral implantation of methylcholanthrene and showed that the C_3H strain reacted more readily than did the other strains studied.

In 1945 Russell showed that the implantation of pellets of methylcholanthrene into the brains of rats fed a standard diet led to the development of gliomata in some 35 per cent of animals and that these animals survived the implantation for an average period of 372 days. He further showed that if following the intracerebral inoculation the rats were rendered deficient in thiamine, riboflavin and niacin then those animals which developed gliomata survived the implantation for an average period of only 270 days. It was in an attempt to establish if a similar phenomenon occurred in C_3H mice and, if so, which vitamin deficiency was responsible that the following experiments were undertaken.

It is well known that patients with malignant disease, including gliomata, show a high globulin-albumen ratio and accordingly the serum of mice carrying intracerebral carcinogen was studied electrophoretically in the hope that some pattern would emerge which would enable the diagnosis of glioma to be made before the animal's death.

MATERIALS AND METHODS

The mice used in these experiments were all of the C_3H strain and were aged between 50 and 70 days at the time of beginning the experiments.

The diet prepared for the animals consisted of protein 19 per cent, fat 5·5 per cent, carbohydrate 65 per cent and in the control diet vitamins A, B, C and D were added in the amounts recommended by Morris (1944).

The mice were divided into 5 equal groups, one was fed the control prepared diet, one an identical diet except that thiamine, riboflavin and niacin were excluded and 3 groups in which only one of the above vitamins was excluded. Those animals in which it was desired to induce vitamin deficiency were initially fed a diet completely lacking in the particular vitamin and when the animals were in an obvious deficiency state sub-optimal amounts of that vitamin were added to the diet.

Pellets of pure methylcholanthrene weighing approximately 1·3 mg. were implanted into the right cerebral hemispheres of 150 mice according to the method described by Seligman and Shear (1939). These animals were equally divided amongst the 5 dietary groups and each group was further sub-divided into 2 sub-groups. One sub-group (*a*) was put on the dietary regime 4 weeks before the intracerebral implantation; in the other the dietary regime was instituted 2 weeks after the intracerebral implantation. Five control groups of 10 animals which did not have the carcinogen implanted were put on one of the above dietary regimes to assess the effect of each deficiency *per se*.

The method of electrophoresis used was the hanging strip method with barbiturate buffer at pH 8·6. Blood for the electrophoresis was obtained by puncturing the conjunctival sinus with fine glass tubing. The serum was allowed to separate at a constant voltage of 180 volts for 16–20 hr. and after fixing and drying the paper was stained with Lissamine Green.

RESULTS

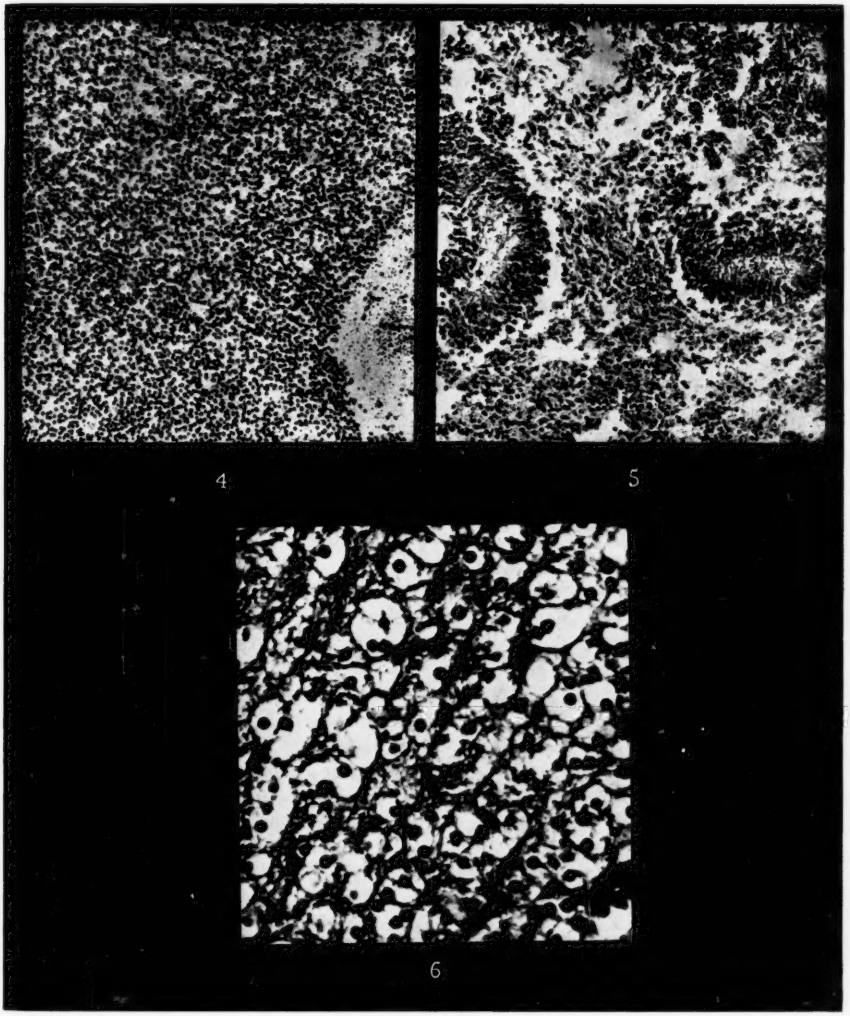
Effect of the dietary deficiency alone

Animals fed the prepared diet with all added vitamins showed no difference in weight from animals fed the standard laboratory diet. Independent of the main experiment 2 male and 4 female mice were fed the complete prepared diet for 6 weeks and then mated. Reproduction and lactation in these females were quite normal. Those animals which were deprived of thiamine showed an initial slight gain in weight lasting 18–20 days and this was followed by a rapid loss of weight so that 30 days after institution of the thiamine-deficient diet the average weight had fallen by 40 per cent. At this stage 4 μg. of thiamine per animal per day was added to the diet. On this regime there was an immediate improvement in the general condition of the animals and their weight gradually increased to 70 per cent of their original weight at which level it was maintained for the duration of the experiment (28 weeks). Animals deprived of riboflavin showed a similar but less rapid response and after 36 days the average weight had fallen by 36 per cent: at this stage 1·5 μg. per animal per day was added to the diet and this was found sufficient to maintain the animals at 70 per cent of their original weight. Animals which were completely deprived of dietary niacin did not show any loss of weight or condition throughout the 28 weeks of this experiment: animals which had been deprived of dietary niacin for 10 weeks were mated and reproduction and lactation were quite normal. Harris and Raymond (1939) found that rats on a diet deficient in niacin continued to thrive and from the above results it would appear that niacin is not an essential dietary requirement for C_3H mice (on the above basic dietary regime). Animals on the dietary regime deficient in thiamine, riboflavin and niacin reacted initially as did those deprived of thiamine alone but at the stage of initial depletion thiamine was sufficient to improve their condition for only a short time and then riboflavin also had to be added to the diet. The amounts of thiamine and riboflavin necessary to maintain the animals on the triple deficiency diet at 70 per cent of their original weight were the same as those needed for the animals on a single deficiency.

EXPLANATION OF PLATES.

FIG. 1.—Spontaneous haemorrhage in right cerebral hemisphere.
FIG. 2.—Astrocytoma right cerebral hemisphere showing terminal haemorrhage.
FIG. 3.—Astrocytoma arising at edge of pellet which is also surrounded by normal brain.
FIG. 4.—Astrocytoma which is very cellular but shows little anaplasia.
FIG. 5.—Ependymoma showing rosette pattern.
FIG. 6.—Oligodendroglioma showing boxing of the nuclei.

Bridges.



4

5

6

The only outstanding autopsy findings in the dietary control animals were in 2 animals which had been on thiamine-deficient regime ; these animals survived the onset of the dietary regime by 10 and 13 weeks respectively and both showed large intracranial haemorrhages (Fig. 1). Intracranial haemorrhage was also noted in 2 animals on the triple deficiency diet which had had intracerebral implants. The haemorrhages were at the site of the implant and had occurred 12 and 14 weeks after the pellet had been inserted. Careful histological study failed to show any evidence of neoplasm at the edges of these haemorrhagic foci.

In none of the dietary control animals nor in those animals on the various dietary deficiencies which had had the carcinogen implanted but did not develop tumour, except for the 4 showing intracranial haemorrhage, was any histological abnormality noted in the brain.

Tumours produced and effect of dietary regime on tumours produced

Of the 150 animals with methylcholanthrene implants 27 developed gliomata and 34 meningeal sarcomata ; all these tumours arose at the site of the implantation.

From the majority of the gliomata the carcinogenic pellet was recovered intact and in those animals which did not develop gliomata only a very slight glial reaction occurred round the pellet. The gliomata varied in size up to 1 cm. diameter and the majority were the seat of recent haemorrhage (Fig. 2). The most striking histological feature was the very close resemblance to the human gliomata (Fig. 3, 4, 5, 6) and all the typical histological patterns of the human gliomata were encountered. This very close histological resemblance of the experimentally produced gliomata and the human gliomata has previously been noted by Seligman and Shear (1939) and Zimmerman and Arnold (1941 and 1943). The 27 gliomata were classified on a histological basis as Glioblastoma multiforme 4, Astrocytoma 11, Oligodendroglioma 2, Ependymoma 4 and mixed tumours 6.

Table I shows the incidence of the gliomata in the various dietary groups. From this Table it is clear that the dietary deficiencies induced did not alter the incidence of the gliomata nor was the incidence altered by instituting the dietary regime before or after the implantation of the carcinogen.

Table II shows the interval elapsing between the implantation of the carcinogen and death in those animals which developed gliomata ; 4 gliomata were discovered when mice which had developed an abnormal albumin-globulin were killed and they are not considered in this Table. The results obtained show that those animals which were chronically deficient in thiamine whether alone or in combination with riboflavin and niacin and which died as a result of a glioma did so significantly earlier than did animals with glioma which were either on a complete diet or on a dietary regime deficient in only riboflavin or niacin.

The meningeal sarcomata showed no difference in incidence in the various dietary groups. There was no significant difference in the survival time of animals with meningeal sarcomata in the various dietary groups, the average figure for the various groups being : complete diet 223 days, diet deficient in thiamine 217 days, diet deficient in riboflavin 206 days, diet deficient in niacin 218 days and diet deficient in all 3 vitamins 237 days.

TABLE I

Dietary group		Number of gliomata	Number of fibrosarcomata
All vitamins added	A	3	3
	B	1	5
	Total	4	8
Deficient in thiamine, riboflavin and niacin	A	3	4
	B	3	3
	Total	6	7
Deficient in thiamine	A	3	3
	B	3	2
	Total	6	5
Deficient in riboflavin	A	4	4
	B	3	4
	Total	7	8
Deficient in niacin	A	2	3
	B	2	3
	Total	4	6

The incidence of intracranial tumours in the various dietary groups. Sub-group A refers to those animals in which the dietary regime was instituted prior to the intracerebral implantation and B to those animals in which the intracerebral implant preceded the institution of the dietary regime.

TABLE II.

	All vitamins added		Deficient in thiamine, riboflavin and niacin		Deficient in thiamine		Deficient in riboflavin		Deficient in niacin	
	A	B	A	B	A	B	A	B	A	B
Interval in days between implantation of pellet and death of animal	249	—	151	182	199	176	230	241	281	292
	291	—	197	206	209	204	292	272	317	—
	321	—	—	239	—	269	322	300	—	—
	—	—	—	—	—	—	330	—	—	—
Average	287	—	174	209	204	216	293	271	299	292
Average in group as a whole	287		195		211		284		297	

Showing the interval between implantation of the carcinogen and death in animals which developed gliomata.

Electrophoretic studies

The electrophoretic patterns obtained were generally satisfactory as regards the separation of the two main protein fractions, *i.e.* globulin and albumin, but the separation of the various globulin fractions was very inconstant. The Albumin/Globulin (A/G) ratios in 100 stock C_3H mice aged between 50 and 300 days were calculated and found to be between 0·75 and 1·35 with a mean reading of 1·08. No variation in the A/G ratio with increasing age was noted. The A/G ratio in the control dietary deficient animals failed to show any significant deviation from

normal even when the animal was in an acute deficiency state. The serum from 11 animals which became suddenly ill and were proven at autopsy to have gliomata was studied. The A/G ratio in these animals varied from 0·35 to 0·55 with a mean reading of 0·5. The A/G ratio in animals with meningeal sarcomata was also found to be consistently low.

The serum of 20 mice with methylcholanthrene implants was studied weekly. Of these animals, 2 which were on the thiamine-deficiency regime, 2 on the riboflavin deficiency and 1 on the thiamine, riboflavin- and niacin-deficiency regime all developed gliomata. Assuming that a reading of 0·6 or below represents an abnormal A/G ratio then an abnormal A/G ratio developed in each of these 5 animals at approximately the same interval after the intracerebral implantation. However, the duration of life following the development of the abnormal A/G ratio was much longer in those animals deficient in riboflavin than in those deficient in thiamine (Table III).

TABLE III

Dietary group	Interval in days between carcinogenic implantation and development of abnormal A/G ratio	Interval in days between carcinogenic implantation and death	Interval in days between development of abnormal A/G ratio and death
Dietary group deficient in riboflavin	154	238	84
	230	322	92
Average	192	280	88
Dietary group deficient in thiamine .	133	175	42
	164	210	46
Dietary group deficient in thiamine, riboflavin and niacin	189	238	49
Average	162	207	46

Showing intervals between implantation of carcinogen, development of abnormal A/G ratio, and death in 5 animals which developed gliomata.

DISCUSSION

In these experiments mice which developed gliomata subsequent to the intracerebral implantation of methylcholanthrene and which were fed a complete prepared diet survived the implantation by an average of 287 days. Mice with similar tumours but fed a diet deficient in thiamine, riboflavin and niacin survived the implantation of the carcinogen by only 195 days. These results agree well with those reported by Russell (1945) who, however, used rats. In examining the survival time of mice with gliomata which were fed a diet deficient only in thiamine or riboflavin or niacin it becomes clear from these experiments that the vitamin deficiency responsible for the shortened survival time was thiamine.

Russell has deduced from his work that the deficiency of thiamine and riboflavin altered the metabolism of the glial cells in such a way as to render these more suceptible to a carcinogenic stimulus. If this is so, then one would only expect that not only would the survival of such mice be reduced but that the incidence of gliomata in the deficient animals would be increased. No difference in the incidence of gliomata in the two groups was, however, noted in the present experiments, nor did Russell find such a difference. These results are, however,

open to a different interpretation; namely that the gliomata arise at approximately the same interval following the implantation of the carcinogen but that the dietary deficiency renders the animals more susceptible to the effects of the glioma. By whatever mechanism the thiamine deficiency causes the shortened survival of animals with gliomata it would appear to be specific to glial neoplasms, since in the present experiments those animals with meningeal sarcomata survived for an equal length of time irrespective of thiamine or riboflavin deficiency. A similar finding was noted by Russell.

In the present experiments on the effect of thiamine deprivation on the central nervous system the only abnormal autopsy finding in those animals which did not have intracerebral implants or did not develop gliomata subsequent to such implants was that 4 such animals showed large intracranial haemorrhages. Dunn, Morris and Dubnik (1947) noted spontaneous intracranial haemorrhage in 30 out of 50 thiamine-deficient C_3H mice and postulated that this was due to a defect in the capillary walls. The outstanding feature on macroscopic examination of the gliomata produced in these experiments, whether the animal was thiamine deficient or not, was that the vast majority of the gliomata had been the seat of terminal haemorrhage, and it would appear that mice with experimentally induced gliomata which are deficient in thiamine die sooner than similar animals on a normal diet because of the greater tendency for haemorrhage to occur into the tumour.

This view is supported by the results of the electrophoretic studies done (Table III). Three mice which were on a thiamine-deficient regime and developed gliomata were having their A/G ratio done weekly: the average interval between the intracerebral inoculation and the development of the abnormal A/G ratio in these animals was 23 weeks and they survived the development of the abnormal A/G ratio by $6\frac{1}{2}$ weeks. Similar figures for the 2 mice not thiamine deficient but in a similar state of inanition due to riboflavin deficiency were: abnormal A/G ratio developed at $27\frac{1}{2}$ weeks and was survived by $12\frac{1}{2}$ weeks. Although the small numbers in this experiment do not permit any firm conclusion to be drawn the results are nevertheless suggestive that the partial withdrawal of dietary thiamine in animals with intracerebral implants of methylcholanthrene does not unduly accelerate the development of gliomata but rather renders the animals more susceptible to the effects of such a tumour.

SUMMARY

The effect of dietary deprivation of thiamine, riboflavin and niacin on the survival time of mice with experimentally induced gliomata was studied.

The gliomata were produced by intracerebral implantation of methylcholanthrene.

It was found that mice which were receiving adequate dietary thiamine irrespective of riboflavin or niacin deficiencies and which developed gliomata survived the implantation of the carcinogen by 287 days whereas those which were deficient in thiamine and died as the result of gliomata did so 211 days after the implantation. The theory is put forward that this is not due to any altered susceptibility to carcinogenic stimulus of glial cells in animals deprived of thiamine, but that these animals die sooner because of the greater tendency for haemorrhage into the substance of the tumour to occur.

I wish to express my deep appreciation to Professor J. H. Biggart for his interest and advice, to Mr. D. McA. Mehaffey for taking the photographs, and to Mr. J. Davidson for his technical assistance.

REFERENCES

ARNOLD, H. AND ZIMMERMAN, H. M.—(1943) *Cancer Res.*, **3**, 682.
DUNN, T. B., MORRIS, H. P. AND DUBNIK, C. S.—(1947) *J. nat. Cancer. Inst.*, **8**, 139.
HARRIS, L. J. AND RAYMOND, W. D.—(1939) *Biochem. J.*, **33**, 2037.
MORRIS, H. P.—(1944) *J. nat. Cancer. Inst.*, **5**, 115.
RUSSELL, W. O.—(1945) *Cancer Res.*, **5**, 140 and 152.
SELIGMAN, A. M. AND SHEAR, M. J.—(1939) *Amer. J. Cancer*, **37**, 364.
ZIMMERMAN, H. M. AND ARNOLD, H.—(1941) *Cancer Res.*, **1**, 919.—(1943) *Amer. J. Path.*, **19**, 939.—(1944) *Cancer Res.*, **4**, 98.

OBSERVATIONS ON LIVING CELLS INFECTED WITH *TOXOPLASMA GONDII**

P. SOURANDER, E. LYCKE AND EBBA LUND

From the Departments of Bacteriology, Pathology and Histology, University of Gothenburg, Sweden

Received for publication September 21, 1959

SABIN AND OLITSKY (1937) demonstrated that *Toxoplasma gondii* was an obligatory intracellular parasite. Guimarães and Meyer (1942) showed that toxoplasma could be cultivated *in vitro* in a wide variety of cells. Since then a number of authors have used the tissue culture technique for various purposes (Mühlpfordt, 1952; Lock, 1953; Chernin and Weller, 1954; Vischer and Suter, 1954; Jacobs, 1956). Although the fundamental facts are well known, information is still lacking concerning several aspects of the behaviour of toxoplasma in tissue cultures, *e.g.* the rate of multiplication, the forming of the cyst-like structures and the reactions of the host cell.

Most of the previous observations seem to have been performed on fixed and stained cultures. In the present study the propagation of toxoplasma was followed in living cells.

MATERIAL AND METHODS

Cancer cells of the Detroit-6 strain and trypsinized human embryo kidney cells were cultivated in a Rose chamber in a liquid medium containing 15 per cent dye-test negative horse or human serum and Hanks' solution with 0·5 per cent lactalbumin hydrolysate at 37°. Well outgrown cultures were inoculated with freshly harvested peritoneal exudate from mice infected with the RH strain of *Toxoplasma gondii*. The inoculated cultures were examined for a period up to 5 days using phase contrast microscopy. The process of infection in a particular cell was followed and photographs were taken with a Hasselblad 500 C. camera. In addition some of the preparations were fixed in Helly's solution and stained with hematoxylin and eosin.

RESULTS

It was found that the toxoplasma parasites might appear intracellularly within 15 min. after the inoculation of the cultures. In spite of the large inoculation doses used (10^6 log units of parasites *i.e.* about 10 parasites per cell) a relatively small number of cells were primarily infected. The reason for this might be that only about 1 per cent of the parasites in the exudate was infectious (Lund, Lycke and Hahn, 1960).

Once inside the cell the parasites increased in size and the first binary division occurred 2–3 hr. after the inoculation. In later stages multiplication proceeded more rapidly, generally in about 1 hr., sometimes even faster.

Many cells were infected by more than one parasite and each one was able to multiply independently. Moreover, cells could be reinfected by new parasites at any stage of the infection (Fig. 1).

* This work was supported by a grant from Statens Medicinska Forskningsråd.

Before multiplication a movement of the parasites towards the nucleus was repeatedly observed and sometimes parasites were found in a perinuclear position (Fig. 2–5). From each parasite a separate clone developed. The appearance of the clones changed according to their orientation in the cytoplasm. Thus they could be shaped like a bunch of bananas or like a rosette. A clear zone was seen around the clones in living cells as well as in fixed and stained preparations of cultures. A similar phenomenon was sometimes observed also for single parasites. In phase microscopy a definite dark boundary was seen around these zones. Inside such a cyst-like structure the parasites multiplied and the size of the parasites decreased as a result of repeated divisions (Fig. 6–8).

Rupture of cells with liberation of numerous parasites was not observed before 2 days after inoculation. These parasites showed active movements particularly during the first minutes after the liberation. Sometimes parasites appeared to be localized in the nuclei (Fig. 9 and 10). Although the cells finally were disrupted the nuclei of the damaged cells still retained their membranes. In fixed preparations the nuclei of heavily infected cells often appeared deformed (Fig. 10).

DISCUSSION

The observations reported in the present study indicate that a few minutes after inoculation of the cultures parasites might be localized intracellularly. Multiplication of parasites could be observed already during the first hours. A slower rate of propagation has been reported by Cross and Anigstein (1957). This might be due to differences in the types of cells and cultivation technique used.

The cells could be reinfected at any stage of the infection and their capacity to afford multiplication of parasites seemed to be limited only by the volume of the host cell. It is thus likely that the factors necessary for the mulplication of the parasites are amply supplied and widely spread throughout the cytoplasm. In our studies both single parasites and groups of toxoplasmas were frequently seen surrounded by clear zones. These were previously observed by Mühlpfordt (1952) and by Jacobs (1956) and described as vacuoles. It might be argued that they are optical artefacts caused by the phase contrast technique. However this seems less plausible as they are found also in fixed and stained preparations. Gustavson, Agar and Cramer (1954) using electron microscopy reported that the space surrounding the parasites contained a fine filamentous or granular substance. In our stained preparations a fine network in the vacuoles was regularly distinguished.

By means of electron microscopy Meyer and de Andrade Mendonça (1957) observed a slight thickening but no distinct cystwall in the cytoplasm surrounding the parasites. In our studies a distinct border was observed on the corresponding place in living as well as in fixed and stained cells.

As claimed by Cross (1947) a tropism of the parasites for the host cell nucleus might exist. We observed repeatedly a trend to perinuclear arrangement of parasites (Fig. 2–5).

In phase contrast microscopy the nuclei usually seemed unaffected but in fixed and stained preparations they often appeared to contain clones or single parasites (Fig. 9–10). Although the parasites appeared to be inside the nucleus it might be argued that they are actually layered on the surface of the nuclear

membrane (Fig. 9). Sometimes darkly stained bodies shaped like small parasites were observed at the same level as the nucleoli. As yet it cannot be stated if these bodies are parasites (Fig. 10).

Preliminary experiments have shown that it might be possible to study the toxoplasma infection of the living cell with time lapse technique. The present study was considered necessary for performing such experiments.

SUMMARY

A study was made with phase contrast microscopy on the propagation of the RH strain of *Toxoplasma gondii* in cultures of cancer cells of the Detroit-6 strain and trypsinized human embryo kidney cells. Particular attention was paid to the different phases and the rate of multiplication of the parasites, their relation to the nucleus and the forming of cyst-like structures in the host cells.

We wish to thank Mrs. Edith Johannsen for skilful technical assistance.

REFERENCES

CHERNIN, E. AND WELLER, T. H.—(1954) *Proc. Soc. exp. Biol., N.Y.*, **85**, 68.
CROSS, J. B.—(1947) *J. infect. Dis.*, **80**, 278.
Idem AND ANIGSTEIN, L.—(1957) Quoted by Thalhammer, O. in ' Die Toxoplasmose bei Mensch u. Tier '. Vienna (Maudrich), p. 18.
GUIMARÃES, F. N. AND MEYER, H.—(1942) *Rev. bras. Biol.*, **2**, 123.
GUSTAVSON, P. V., AGAR, H. D. AND CRAMER, D. I.—(1954) *Amer. J. trop. Med.*, **3**, 1008.
JACOBS, L.—(1956) *Ann. N.Y. Acad. Sci.*, **64** (2), 154.
LOCK, J. A.—(1953) *Lancet*, **264**, 324.
LUND, E., LYCKE, E. AND HAHN, E.—(1960) *Acta path. microbiol. scand.*, **48**, 99.
MEYER, H.—(1949) ' The Cultivation of Protozoa in Tissue Cultures '. Publ. of the Instituto de Biofisica, Universidade de Brasil. (Rio de Janeiro.)
Idem AND DE ANDRADE MENDONÇA, I.—(1957) *Parasitology*, **47**, 66.
MÜHLPFORDT, H.—(1952) *Z. Tropenmed. u. Parasit.*, **4**, 64.
SABIN, A. B. AND OLITSKY, P. K.—(1937) *Science*, **85**, 336.
VISCHER, W. A. AND SUTER, E.—(1954) *Proc. Soc. exp. Biol., N.Y.*, **86**, 413.

EXPLANATION OF PLATE

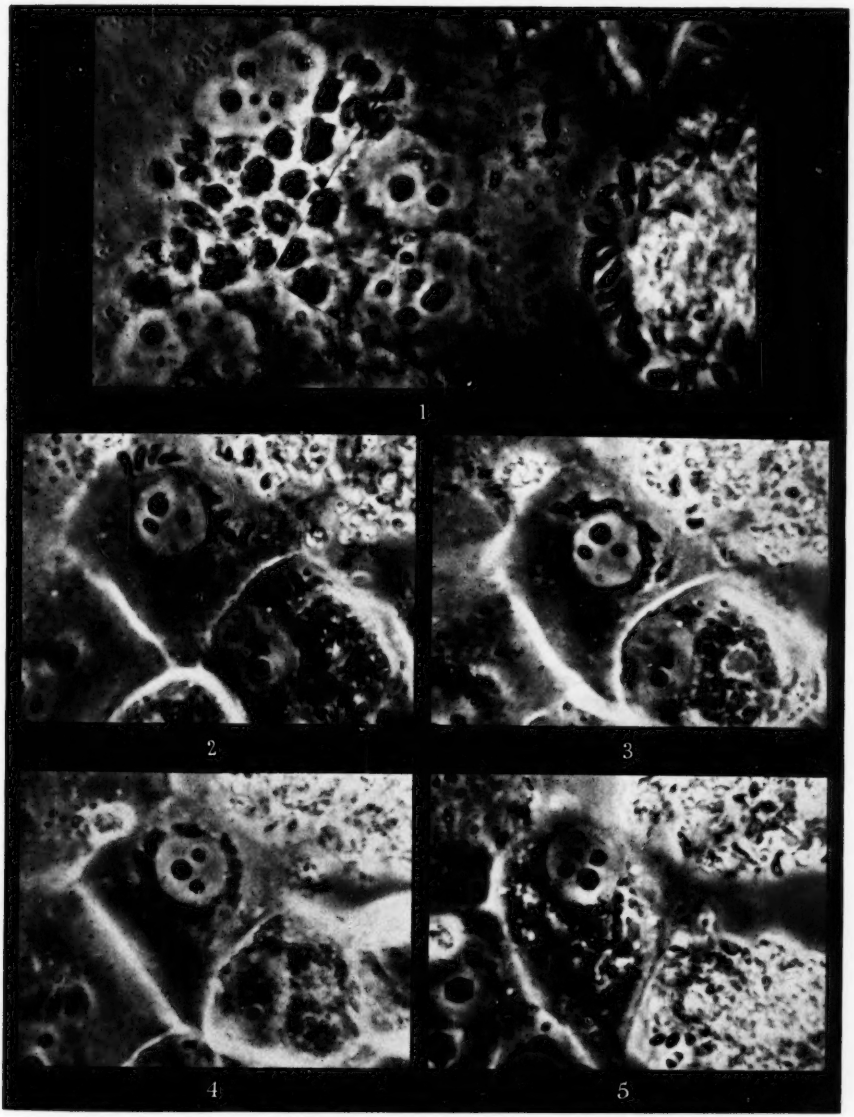
FIGS. 1–8.—Phase contrast photographs of living cells in tissue culture. Objective : Apo 40/0·07. Magnification about × 720.

FIG. 1.—Multinuclear giant cell of the Detroit-6 strain 5 days after the inoculation. Several clones of parasites are seen between the nuclei. On the right hand reinfection of the cell with parasites recently liberated by the bursting of an adjacent cell.

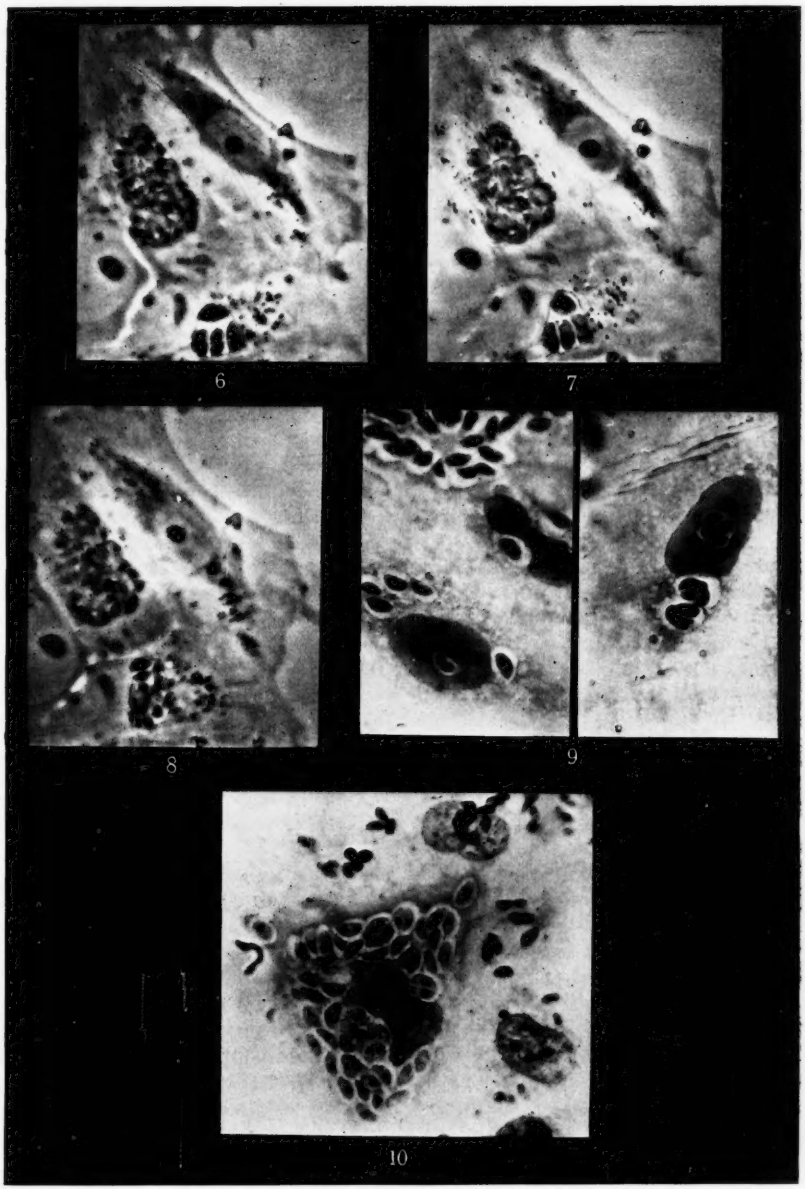
FIG. 2–5.—Kidney cells of human embryo 2 days after inoculation. The cell in the centre contains parasites which gradually become localized in a perinuclear position. Time interval from Fig. 2 to 4, three hours. Three hours later (Fig. 5) multiplication has occurred and the nucleus is dislocated. The cell in the lower corner at the right which in Fig. 2 contains a cluster of parasites has in Fig. 5 ruptured and liberated numerous parasites.

FIG. 6–8.—Kidney cells one day after inoculation. Parasites in cyst-like structures of which the lower one is limited by a definite dark boundary (Fig. 6). Ten minutes later the parasites have changed somewhat in appearance (Fig. 7). In the following figures (Fig. 8) multiplication has definitely occurred. Note the size of the newly formed parasites compared with the original ones.

FIG. 9–10.—Human embryo kidney cells fixed in Helly's solution and stained with H. and E., × 700. In Fig. 9 a single toxoplasma and a clone of parasites are seen in the middle of nuclei. In Fig. 10 several deeply stained bodies resembling parasites appear inside the nucleus. The significance of these findings is discussed in the text.



Sourander, Lycke and Lund.



Sourander, Lycke and Lund.

THE ACTION OF THE THERMOLABILE TOXIN OF *SHIGELLA DYSENTERIAE* ON CELLS CULTIVATED *IN VITRO*

G. VICARI, A. L. OLITZKI* AND ZAHAVAH OLITZKI†

From the Laboratorio di Microbiologia, Istituto Superiore di Sanita, Rome, Italy

Received for publication September 23, 1959

SINCE Levaditi and Muttermilch (1913) demonstrated the action of diphtheria toxin and snake poison on fibroblast-like cells around fragments of chicken embryo heart and their neutralization by specific antitoxins, many authors have confirmed that various bacterial toxins inhibit the growth of tissue cells in cultures and that their toxin action can be neutralized by specific antitoxins. The literature on these earlier investigations was reviewed by Penso and Vicari (1957).

Penso and Vicari (1957*a*, *b* and *c*; 1959) carried out detailed investigations taking into consideration the specific action of each toxin on plasma and nuclei of cells, the minimal incubation time necessary for the production of the pathological changes, the stepwise progress of the process and its final outcome. Their experiments described the specific effects of diphtherial and staphylococcal (1957) and clostridial toxins (1959) on tissue cultures and their neutralization by antitoxins.

Lennox and Kaplan (1957) showed that HeLaS-3 cells were susceptible to diphtheria toxin. Furthermore, Kaplan and Lennox (1958) showed that only one molecule (or a few) of diphtheria toxin are necessary to destroy the colony forming ability of already attached HeLaS-3 cells.

Lawrence (1959) investigated the effect of staphylococcal α-toxin, streptolysin O and streptolysin S on skin respiration and on migration and viability of epithelial cells *in vitro*. Staphylococcal α-toxin was found to be extremely toxic, streptolysin O was less so, while streptolysin S seemed to be comparatively non-toxic to skin cultures.

The purpose of the experiments described below was to investigate the action of dysentery toxin on cells in tissue cultures. This work seemed to be worthwhile in view of the discussion, whether the toxin of *Shigella dysenteriae* (Shiga) is a real neurotoxin, or whether it is a toxin which damages different types of cells and produces neurological disabilities as secondary effects of these damages. In the work done by van Heyningen and Gladstone (1953) and by van Heyningen (1955) on production, purification of the toxin and metabolic processes related to its production it is designed as "neurotoxin", while in earlier communications as that of Steabben (1943) the name "neuro-enterotoxin" is used. Koch and Olitzki (1946) reported that the paralytic symptoms, oedemas and haemorrhages of the intestines were produced in rabbits exclusively by the thermolabile toxin of *Sh. dysenteriae*, but never by products of other enteric pathogens prepared by the same method. The Shiga-toxin produced frequent haemorrhages with the

*† Present address: Department of Bacteriology, The Hebrew University-Hadassah Medical School, P.O.B. 1172, Jerusalem, Israel.

paralytic symptoms. However, this simultaneous appearance of haemorrhages was irregular and could never be predicted. Therefore, they concluded that the same thermolabile toxin may produce these haemorrhages in addition to the paralytic symptoms, probably as result of a synergistic action with other bacterial products.

Howard (1955) described the dysentery toxin as neurotoxin. However, he concluded from the results of his experiments, that neuronal destruction by the toxin cannot be attributed to a direct action of the toxin on the nerve cell. Bridgwater, Morgan, Rowson and Payling Wright (1955) demonstrated that the intravenous injection of a lethal dose of purified Shiga toxin in rabbits is followed by the appearance of progressively increasing focal haemorrhages in the grey matter of the spinal cord enlargements. These extravasations of blood first arise long before the animals present any neurological disability and precede by many hours the appearance of injury to the nerve cell of the spinal motor nuclei. The authors concluded, therefore, that the Shiga toxin in distinction to botulinus or tetanus toxin may be regarded as a secondary neurotoxin.

The plan of the following work was, therefore, as follows: the action of the toxin exerted by the Bukarest R-strain, which produces the thermolabile toxin without producing the thermostabile endotoxin (Boivin and Mesrobeanu, 1937; Olitzki, Bendersky and Koch, 1943) was investigated *in vitro* by the aid of different cell strains. Its mode of action, its neutralization by specific antitoxin and toxoidization was studied. Finally the action of the crude toxins was compared with that of a purified product.

MATERIALS AND METHODS

Preparation of the toxin of Sh. dysenteriae

Sh. dysenteriae, strain Bukarest R, was inoculated in nutrient broth and incubated for 48 hr. Sixty Roux bottles containing nutrient agar were inoculated each one with 8 ml. of the 48 hr. broth cultures. After 48 hr. incubation at 37° the bacteria were washed off with saline, kept overnight in the refrigerator, where they sedimented, and the supernatant fluid discarded. The sedimented bacteria were again resuspended in saline, centrifuged, the supernatant fluid discarded and the sediment frozen and exposed to the action of a disintegrator twice for 3 min. and a third time for 15 sec. The resulting frozen powder was kept overnight in the refrigerator, and on the following day after thawing a slimy paste was formed. Microscopically Gram-negative amorphous material with few intact bacteria were observed. This paste was suspended in 200 ml. of saline, shaken at room temperature, until a fine suspension was formed and then centrifuged at 3000 r.p.m. for 30 min. This procedure was repeated 5 times. However, the examination of the resulting supernatant fluids showed, that only the first and the second one were really toxic, the third one was slightly toxic and the others were completely non-toxic. Therefore, only the first and second supernatants were used as toxins.

Properties of the toxins employed

As it was expected from the R-strain, the bacterial extracts contained only the thermolabile toxin and were completely devoid of the thermostable endotoxin. Heating of the extracts at 100° for 30 min. destroyed completely their toxic activities so that even 0·5 ml. of the heated toxin could be injected intraperitoneally into mice without lethal effects. When the first and second extracts were heated for 10 min. at 58° they lost about 50 per cent of their toxicity. A precipitate appeared which after centrifugation and repeated washings in saline proved to be non-toxic. The supernatant fluids still retained a part of their toxicity. Some of the properties of the 4 resulting toxic products, not heated and heated for 10 min. at 58° are summarized in Table I.

TABLE I.—*Toxicity and Nitrogen Content of Four Toxins*

Toxin No.	Method of preparation	Nitrogen (μg./ml.)	LD_{50} intraperitoneally for white mice (ml.)	Minimal cytopathogenic dose (ml./ml. culture fluid*)
1	First extraction of disintegrated bacteria	854	4×10^{-3}	1×10^{-6}
2	Second extraction of disintegrated bacteria	430	9×10^{-3}	4×10^{-6}
3	First extract heated at 58° for 10 min., insoluble precipitate removed by centrifugation	651	6×10^{-3}	1×10^{-6}
4	Second extract treated as No. 3	155	1×10^{-2}	4×10^{-6}

* The minimal cytopathogenic dose (MCpD) was the same in experiments with KB cells, human liver and monkey kidney cells.

Table I shows that all the toxins derived from disintegrated bacteria exerted still a strong cytopathogenic effect in a concentration of 1×10^{-6}–4×10^{-6} ml./1 ml. of culture fluid. In this respect the toxins were more active than the supernatant fluid of the culture which in spite of its nitrogen content of 890 μg./ml. exerted a cytopathogenic effect only in a concentration of 1×10^{-3} ml./ml. culture fluid.

A further concentration and purification of toxin No. 3 was carried out by repeated dialysis as described by van Heyningen and Gladstone (1953). The results obtained with this product will be summarized below.

Antitoxins for neutralization experiment

As antitoxin we employed a standard antiserum distributed by the State Serum Institute, Copenhagen. This serum contained 200 International Units/ml. Two rabbit immune sera were prepared by the immunization of rabbits with two different antigens, the first one—bacteria washed by saline and dried *in vacuo*, the other one—the bacterial residue after the extraction of the toxin described above. The rabbits received 4 times injections of these antigens at 8 day intervals. Each time 1·0 ml. containing 10 mg. of dried bacterial substance was administered intracutaneously at various sites of the skin. Blood was taken 15 days after the last injection. When it became clear that normal calf sera contain dysentery antitoxin normal calf sera inactivated at 56° for 30 min. were employed in neutralization tests.

Preparation of cell cultures for testing the cytopathogenic effect of dysentery toxin

KB cells.—A cellular suspension was prepared from a bottle of the collection 24 hr. before the start of the experiment. The cells were detached from the glass by trypsinization. A 0·06 per cent trypsin Difco solution in saline A according to Puck was employed. After the detachment of the cells the trypsin was eliminated by centrifugation at 500 r.p.m. for 1 min. The supernatant was discarded and the sedimented cells resuspended in the growth medium consisting of Hanks' saline 85 per cent, Lactalbumin hydrolysate (solution 5 per cent) 5 per cent and calf serum 10 per cent. The suspension was adjusted in order to obtain about 2×10^5 cells/ml. and was distributed in tubes in portions of 0·5 ml./tube. The tubes were hermetically closed with rubber stoppers and incubated at 37° for 24 hr. Then the growth medium was removed and replaced by 1·5 ml. of a maintenance medium consisting of Hanks' saline 88 per cent, Lactalbumin hydrolysate (solution 5 per cent) 10 per cent, calf serum 2 per cent.

The toxin dilutions or the toxin-antitoxin mixtures were prepared in Hanks' solution in such a way that the required quantities were present in 0·5 ml. After addition of this amount to 1·5 ml. of the maintenance medium the total volume of fluid present in the culture tube was 2·0 ml.

Human liver cells.—The same method as for the examination of the KB cells was followed. The only difference was the composition of the growth medium : Hanks' solution 70 per cent, Lactalbumin (5 per cent solution) 10 per cent, calf serum 10 per cent, horse serum 10 per cent.

Preparation of cells for a toxin fixation experiment

The growth medium was removed from 3 tubes containing 24 hr. cultures of KB cells, and 2 ml. of a solution containing 0·02 g./l. sodium versenate were added to each tube. After an incubation at 37° for 10 min. the suspensions of the detached cells were centrifuged at 500 r.p.m. for 1 min. Then the versenate solution was discarded and the sedimented cells harvested from 3 tubes resuspended in 2·9 ml. of the same maintenance medium as described above. The total volume of the suspension amounted to 3 ml., and 1 ml. contained the cells harvested in the average from one tube.

The cell concentration was determined in Buerker's haemacytometer. The cell count was performed on 4 drops and for each drop on all the 9 squares of the chamber. The mean was in one experiment $2{\cdot}0 \times 10^5$ cells/ml. with a standard error $1{\cdot}26 \times 10^4$, and in another experiment $2{\cdot}1 \times 10^5$ ($\pm$ $4{\cdot}0 \times 10^4$) cells/ml.

In order to obtain greater cell concentrations and to exclude the calf serum present in the maintenance medium the cells were detached by versenate from 12 tubes. They were resuspended after two centrifugations at 500 r.p.m. for 1 min. in 1·2 ml. of the following medium: Hanks' solution 88 per cent, Lactalbumin (5 per cent solution) 10 per cent, protein-free ultrafiltrate of calf serum (Difco) 2 per cent. 0·2 ml. were used for cell counting. For this purpose the original cell suspension was diluted 1 : 10 with Hanks' solution. The original suspension contained in 1 ml. an average of $1{\cdot}09 \times 10^6$ cells with a standard error of the mean corresponding to $1{\cdot}14 \times 10^5$.

RESULTS

Mode of action of the toxin on the cells

When KB cells were brought in contact with the Shiga-toxin, then the first changes in the culture were observed under a low magnification after a 4-hr. contact. The presence of at least 10 MCpD/ml. was required for this relatively quick appearance. When only 1 MCpD/ml. was present, then the first pathological changes appeared after 6 hr.

The histological examinations of the cultures after different time intervals revealed that already after 30 min. contact with 100 MCpD of toxin some cells present a transparent cytoplasm and thickened cell membranes, while others show at their periphery and within the cytoplasm large eosinophilic granules, as shown in Fig. 2.

This process continued, and after a 3-hr. contact with the toxin practically all cells were damaged; they were swollen with a transparent protoplasm and thickened cell membrane or filled with granules and often consisting of a mass of granules around a pyknotic nucleus, as presented in Fig. 3 and 4.

This degeneration process leads to a destruction of the majority of cells, which is almost completed after 6 hr. contact with the toxin, as presented in Fig. 5, and 6.

Neutralization of the toxin by specific antitoxin

Preliminary experiments showed that it was impossible to neutralize the cytopathogenic action of the dysentery toxin on tissue cells, when toxin and antitoxin were separately added to the culture fluid. Experiments of this kind were carried out with concentrations of 0·01 and 0·001 ml. toxin/ml. culture fluid and with varying quantities of antitoxin up to 10 I.U./ml. of culture fluid. It seemed, therefore, that the very short time of contact between the toxin and the cells prior to its complete neutralization by the antitoxin was sufficient in order to produce the typical cytopathogenic effect.

We changed, therefore, the technique and kept the toxin-antitoxin mixtures in Hanks' solution for 1 hr. at 37° and then overnight in the refrigerator. Then 0·5 ml. of the toxin-antitoxin were added to 1·5 ml. of the culture fluid, giving the final concentrations of toxin and antitoxin mentioned in Table II.

TABLE II.—*Neutralization of Dysentery Toxin by Antitoxin Demonstrated in Tissue Cultures*

Final concentration of toxin per ml. of culture fluid (ml.)	KB cells (I.U./ml. of culture fluid)			Liver cells (I.U./ml. of culture fluid)	
	0	0·5	5·0	0	0·5
0·01	+	+	+	+	+
0·005	+	+	−	+	±
0·0025	+	±	−	+	±
0·0020	+	±	−	+	±
0·0015	+	±	−	+	±
0·0010	+	±	−	+	±
0·0005	+	−	−	+	−
0·00025	+	−	−	+	−
0·0001	+	−	−	+	−
0·00001	+	−	−	±	−
0·000005	−	−	−	−	−
0·0000025	−	−	−	−	−

+ = All cells damaged.
± = Cells partly damaged, partly normal.
− = Cells normal.

Table II shows that 0·5 I.U. neutralized completely 50 MCpD and 5·0 I.U. 500 MCpD. The experiment shows, furthermore, that the results obtained with KB cells and normal liver cells wre almost identical.

Table III shows the corresponding neutralization test *in vivo*. According to the results of this test the L_0 was 0·01, the L_+100 per cent 0·04, and the L_+50 per cent 0·025 ml.

TABLE III.—*Neutralization Test* in vivo *with the same Toxin and Antitoxin as Employed in the* in vitro *Experiment Presented in Table II**

Quantity of toxin present (ml.)	Deaths occurring	Average time of deaths after injection (days)	Accumulative numbers			Percentage of survivors
			Died	Survived	Total	
0·01	0	—	0	10	10	100·0
0·02	2	4·5	2	5	7	71·4
0·03	3	4·0	5	2	7	28·6
0·04	5	3·6	10	0	10	0
0·05	5	3·4	15	0	15	0
0·06	5	3·2	20	0	20	0
0·07	5	3·0	25	0	25	0

* One I.U. added to varying quantities of toxin injected intraperitoneally in mice weighing 20 g. (Total volume 1 ml. per mouse.) Groups of 5 mice.

Comparing the results of both tests it can be concluded that as a result of the high sensitivity of the cell cultures to the action of the toxin the amounts of antitoxin required for a complete neutralization *in vitro* are higher than those

required *in vivo*. The L_0 obtained *in vitro* with 5 I.U. is very close to that obtained with 1 I.U. *in vivo*, 0·005 and 0·01 respectively.

By the *in vitro* method with KB cells we examined also the neutralizing effect of normal calf sera and rabbit immune sera, which were prepared by immunization with dried bacteria and with the bacterial residue after extraction as described above. Table IV shows that 3 samples of calf sera diluted 1 : 40 exerted in the culture antitoxic effects which corresponded in sera No. 1 and No. 3 with the neutralizing effect of 0·5 I.U./ml. culture fluid and in serum No. 2 with 0·05 I.U./ml. culture fluid, *i.e.* the undiluted calf sera No. 1 and 3 contained 20 I.U./ml. and the calf serum No. 2 contained 2 I.U./ml.

TABLE IV.—*Neutralization of Dysentery Toxin by Normal Calf Sera, Immune Rabbit Sera and Varying Concentrations of the Standard Antitoxin*

	Cytopathogenic effect								
		In the presence of							
		Standard antitoxin (I.U./ml. culture fluid)			Normal calf sera (0·025 ml./ml. culture fluid)			Rabbit immune sera (0·025 ml./ml. culture fluid)	
Shiga-toxin MCpD/ml. culture fluid	Without antitoxin	0·05	0·5	5·0	No. 1	No. 2	No. 3	No. 1	No. 2
1000	+	+	+	+	+	+	+	+	+
100	+	+	±	−	+	+	+	±	−
10	+	±	−	−	−	±	−	−	−
1	+	−	−	−	−	−	−	−	−

The 2 rabbit immune sera contained a greater amount of antitoxin. Serum No. 1 prepared by immunization of rabbits with dried bacteria exerted in dilution 1 : 40 in the culture fluid an antitoxic effect corresponding to 0·5 I.U./ml. and antiserum No. 2 prepared by immunization with disintegrated and extracted bacteria an antitoxic effect corresponding to 5 I.U./ml. Since both sera were present in a dilution 1 : 40 it follows that serum No. 1 contained 20 I.U. and No. 2 serum 200 I.U./ml. like the standard serum. It seems, therefore, that intact bacteria are less suitable antitoxin producing antigens than disintegrated bacteria.

Fixation of the toxin by the cells

Cultures of KB cells were exposed to the action of graded concentrations of toxin No. 2 for varying periods of time. Then the toxin was removed and the culture washed 3 times with Hanks' solution. One group of the cultures was now covered with 2·0 ml. of the maintenance medium, while a corresponding group was covered with the same medium, to which 1 I.U./ml. of dysentery antitoxin was added. After a 24 hr. incubation the results were observed which are summarized in Table V.

Table V shows that a contact as short as 5 min. with 10 or 100 MCpD/ml of toxin was sufficient to provoke well marked cytopathogenic effects. The replacement of the toxin by the plain medium or by the medium which contained antitoxin could not prevent the appearance of the pathological changes. The question had arisen as to whether this irreversible process has to be ascribed to an early fixation

TABLE V.—*The Effect of Short Contacts* (5–240 *min.*) *of KB Cells with Dysentery Toxin*

Time of exposure to toxin (min.)	Results obtained with different concentrations of toxin (ml./ml. of culture fluid)			
	0·001		0·0001	
	M.	A.T.	M.	A.T.
5	+	+	+	+
10	+	+	+	+
15	+	+	+	+
30	+	+	±	±
60	+	+	±	±
120	+	+	±	±
180	+	+	±	±
240	+	+	+	+

M. = Replacement of the toxin by the maintenance medium.
A.T. = Replacement of the toxin by the same medium containing 1 I.U./ml. antitoxin.

of the toxin by the cells, which is completed within 5 min., while the first visible pathological changes used to appear after 4 hr. For this purpose we added graded quantities of the toxin containing culture fluids, which in the former experiment were removed from the cultures after varying intervals, to fresh cell cultures. The toxic results obtained with them are represented in Table VI.

TABLE VI.—*The Loss of Toxicity of Culture Fluids which Contained at the Beginning of the Experiment* 100 *MCpD/ML. of Dysentery Toxin. Calf Serum Present in the Medium*

Time of exposure to cell culture (min.)	Results observed after contact with cells			
	Culture fluid undiluted	Culture fluid diluted		
		1 : 5	1 : 50	1 : 100
10	+	±	−	−
15	+	±	−	−
30	(±)	−	−	−
60	(±)	−	−	−
120	(±)	−	−	−
180	(±)	−	−	−
240	(±)	−	−	−
Toxin not exposed	+	+	+	+

+ = Positive after 24 hr.
± and (±) = Positive after 48 hr.

Table VI shows that the culture fluids which had contained in the beginning of the experiment 0·001 ml., *i.e.* 100 MCpD/ml. had after the short contact with the cells lost almost completely their toxicity. This process was a quick one and was almost completed after 10 min.

In order to ascertain whether the toxin was really fixed by incubation with the cells and not by the calf serum present in the medium we prepared a cell suspension as described above. Twofold graded dilutions of this suspension ranging from 2×10^5–4×10^2 cells/ml. were brought in contact with 10 MCpD/ml. toxin in the maintenance medium centrifuged and the supernatant added to

fresh cell cultures. All the toxin samples which were formerly in contact with the cells and also a control tube, where the toxin was in contact with the maintenance medium without the presence of the cells, proved to be completely non-toxic. The detoxication was, therefore, not ascribed to the cells, but to the calf serum present in the culture medium. Different samples of calf serum employed for the cultures were, therefore, examined for their antitoxic activity in comparison with a standard antitoxin. The results of this experiment were summarized in Table IV.

In order to exclude the antitoxic effect of the calf serum present in the maintenance medium the toxin fixation experiments were modified. Instead of the regular maintenance medium which contained calf serum, the cells were suspended in a medium which contained 88 per cent Hanks' solution, 10 per cent Lactalbumin hydrolysate (5 per cent solution) and 2 per cent calf serum ultrafiltrate (Difco). Serial dilutions of the cells were prepared with this medium ranging from $3{\cdot}0 \times 10^5$ $-3{\cdot}75 \times 10^2$, To these cell suspensions portions of 1 ml. of medium were added which contained 40 MCpD/ml. of toxin, so that the final concentration of the toxin was 20 MCpD/ml. After 30 min. contact at 37° the cells were removed by centrifugation at 750 r.p.m. for 1 min. and the clear supernatant fluids added to fresh cell cultures. After an interval of 5 min. 2 per cent calf serum was added to each culture, in order to prevent the neutralization of the toxins before their action on the cells. The experiment showed that even a strong cell suspension containing 3×10^5 cells/ml. was unable to inactivate the toxin after a 30 min. contact at 37°.

We repeated, therefore, the experiment with a more concentrated cell suspension which contained $1{\cdot}09 \times 10^6$ cells/ml. (standard error $\pm 1{\cdot}1 \times 10^5$). After an incubation of 30 min. at 37° in a shaking apparatus the cells were removed by centrifugation and the supernatant fluid examined for its toxin content, in comparison with a corresponding portion which was not brought in contact with the cells. The same precautions as in the former experiment were maintained, in order to exclude antitoxic effect of the calf serum. The results of this experiment are summarized in Table VII. The Table shows that by the contact of the toxin with about 10^6 cells a part of the toxin was inactivated.

Detoxication experiments

Varying concentrations of formaldehyde ranging from 0·8 to 0·04 per cent were added to equal portions of toxin. These mixtures were incubated at 37° and after intervals of 12 and 24 days their toxicity was tested *in vivo* and in

EXPLANATION OF PLATE

FIG. 1.—KB cells. Control culture without toxin. H. and E. ×225.

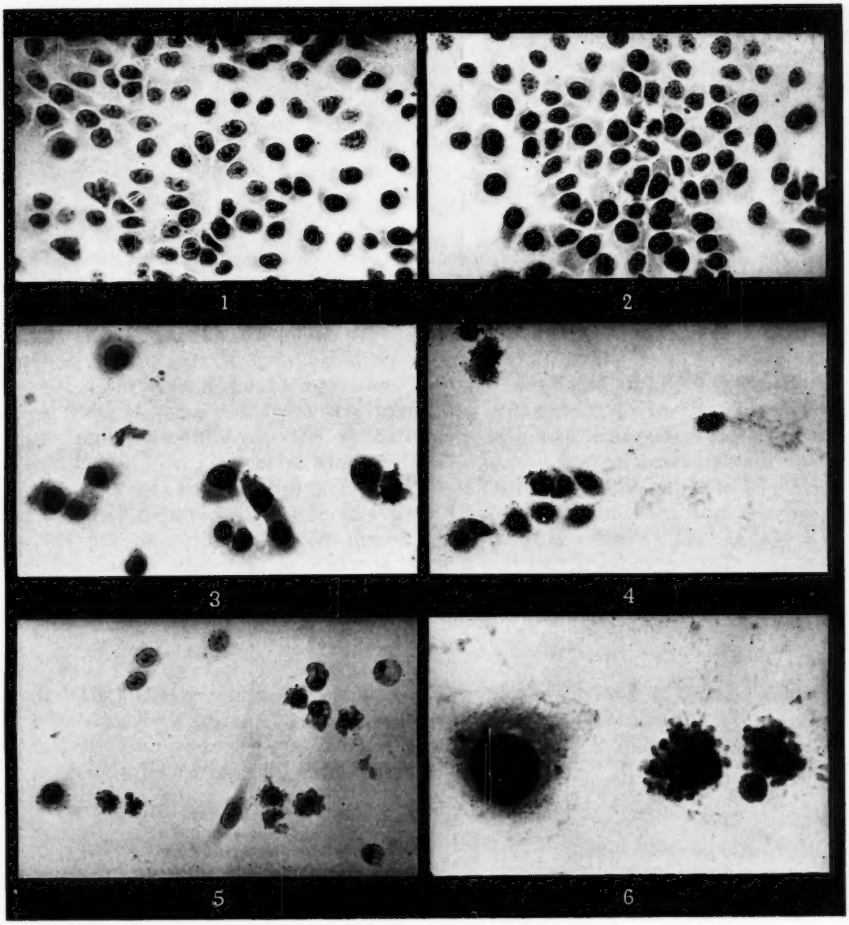
FIG. 2.—KB cells after 1 hr. contact with 100 MCpD of Shiga-toxin. Several cells are damaged. Some of them present a transparent cytoplasm and thickened membrane. Others present at the periphery eosinophilic granules. H. and E. ×225.

FIG. 3.—KB cells after 2 hr. contact with 100 MCpD of Shiga-toxin. All cells are damaged. One may see also cell debris. H. and E. ×225.

FIG. 4.—KB cells after 3 hr. contact with 100 MCpD of Shiga-toxin. Cells with granules and nuclear pyknosis; there is a lot of cell debris. H. and E. ×225.

FIG. 5.—KB cells after 6 hr. contact with 100 MCpD of Shiga-toxin. The cells are heavily damaged. The nuclear pyknosis is more evident. H. and E. ×225.

FIG. 6.—Details of Fig. 5. H. and E. ×530.



Vicari, Olitzki and Olitzki.

TABLE VII.—*The Partial Inactivation of Dysentery Toxin by Contact with KB Cells at 37° for 30 min. Toxin (0·0004 ml.) Incubated with 10^6 Cells. Calf Serum Not Present.*

Dilutions of the toxin in culture fluid	Cytopathogenic effects observed with toxin	
	Incubated with KB cells	Not in previous contact with KB cells
Undiluted	+	+
1 : 10	±	+
1 : 20	±	+
1 : 40	−	±
1 : 80	−	−

tissue cultures. In order to exclude the toxic effect of formaldehyde samples taken for examination were subjected to dialysis against physiological saline. The results of this examination were presented in Table VIII. They show that after 12 days' incubation none of the samples was completely detoxicated. However, after 24 days' incubation the detoxication of the sample which contained 0·8 per cent formaldehyde was *in vivo* completed, while by the tissue culture method some rests of active toxin was still detected. In the presence of 0·4 per cent formaldehyde the detoxication was not complete, as indicated by both the *in vivo* and the *in vitro* test. The former test indicated active toxin present in 0·2 ml., the latter one in 0·002 ml., *i.e.* in an amount which is 100 times less than that required for the former test. Concentration of formaldehyde lower than 0·4 per cent did not exert marked detoxicating effects. The MCpD was somewhat higher and deaths occurred with a certain delay in comparison with those occurring after injection of untreated toxin, which indicates that during the time of incubation with formaldehyde some slight detoxication has taken place.

TABLE VIII.—*Detoxication Experiments. The Toxic Actions of Toxin Samples Incubated for Different Time Intervals with Varying Concentrations of Formaldehyde. Comparison between the Cytopathogenic Effect and the Lethal Effect on Mice after Intraperitoneal Injection*

Formaldehyde concentration (per cent)	Minimal cytopathogenic concentrations (ml. toxin/ml. culture fluid)			Lethal effects on mice after incubation			
	After 12 days (37°)		After 24 days (37°)	12 days (ml.)	24 days (ml.)		
	KB cells	Liver cells	KB cells	0·1	0·5	0·2	0·1
0·8	0·001	0·001	0·02	D. 7 day	S.	S.	S.
0·4	0·0001	0·001	0·002	D. 6 day	—	D. 4 day	S.
0·2	0·00001	0·0001	0·00002	D. 5 day	—	—	D. 5 day
0·08	0·00001	0·00001	0·00001	D. 4 day	—	—	D. 4 day
0·04	0·00001	0·00001	0·00001	D. 4 day	—	—	D. 3 day
Toxin control	0·000005	0·00001	0·000005	D. 3 day	—	—	D. 3 day

D. = Deaths observed.
S. = Survived.

Results obtained with a purified toxin

By repeated precipitation following dialysis and solution of the precipitated material in a 1 per cent NaCl solution a toxin solution was obtained which in a concentration of 10^{-9} ml./ml. of culture fluid exerted a marked cytopathogenic effect. When some insoluble material were removed by centrifugation the supernatant fluid still retained the same MCpD. The insoluble material was repeatedly washed and resuspended in saline. It proved to be of relatively low toxicity; its MCpD was 10^{-5} ml./ml. of culture fluid. Since the finally purified toxin solution contained 1 mg./ml. of dried substance the MCpD of this purified toxin was 0·001 mμg./ml. of culture fluid. When this toxin was injected intraperitoneally into mice the LD_{50} was 0·5 μg.

DISCUSSION

The experiments reported above showed that the thermolabile toxin of *Sh. dysenteriae* exerts a strong cytopathogenic effect on different strains of cells and that these cells did not differ in their sensitivity towards the action of the toxin. KB cells, human liver and monkey kidney cells acted in the same manner. The changes which were produced by this toxin were specific and in none of the experiments done by different authors with other bacterial toxins were similar changes observed. The action of the toxin is quick and a short contact with the cells, probably less than a few minutes, was sufficient to bring the cells to a complete degeneration. However, the cultures of KB cells gave mostly more uniform results than the other cultures. Liver and kidney cell cultures contained cells which differed in their sensitivity towards the Shiga-toxin showing under the same conditions damaged and normal cells together. It seems, therefore, that KB cells, which were isolated and described by Eagle (1955), present an excellent object for the study of Shiga-toxin. Antitoxin, when administered simultaneously but separately. was ineffective, while by preincubation of the toxin with the antitoxin and the addition of the neutral mixture to the culture the antitoxic effect of sera was observed. There was a complete correlation between the lethal action of the toxin *in vivo* and the cytopathogenic effect *in vitro*. Since the quantity of crude toxin required for the production of the cytopathogenic effect was about 3700 times less than that required for the 50 per cent lethal effect in mice, it became evident that the *in vitro* test presents a much more delicate tool in studies with the Shiga-toxin. In studies on toxoidization it indicates the presence of rests of toxin, where the *in vivo* test indicates a complete detoxication. Furthermore, with the aid of very small amounts of toxin low concentrations of antitoxin could be detected, even in normal calf sera, where their presence was not expected. Moreover, the deaths after administration of Shiga-toxin in mice occur only several days after the injection and after administration of small doses even after 7 days. On the other hand, the *in vitro* test shows the first results after 4–6 hr. and the final reading can be done after 20 hr. We have, therefore, the possibility to answer the question whether a toxin is completely toxoidized or whether a serum still contains antibodies within one day.

SUMMARY

The thermolabile toxin of *Sh. dysenteriae*, named by many authors the neurotoxin, exerts specific cytopathogenic effects on cells in tissue cultures. KB cells,

normal human liver and monkey kidney cells showed the same degenerative processes, starting with thickening of the cells' membrane and ending with a complete destruction of the cytoplasm and pyknosis of the nucleus.

The quantities of crude toxin required for this effect are about 3700 less than those required for the production of 50 per cent lethal effects in mice when administered intraperitoneally. A purified toxin exerted its cytopathogenic effects when 0·001 mμg./ml. of culture fluid were present. The cytotoxic factor was toxoidized by formaldehyde in suitable concentrations together with the lethal factor *in vivo*, it was neutralized by antitoxin and concentrated and purified by repeated dialysis against distilled water. Both factors resisted heating at 56°, but were inactivated by boiling. The action of the toxin on the cells was a quick one, replacement of the toxin by antitoxin in the tissue culture, even a few minutes after its contact with the cells, did not prevent the process leading to destruction of the cells.

REFERENCES

Boivin, A. and Mesrobeanu, L.—(1937) *C.R. Soc. Biol., Paris*, **124**, 222, 323 and 442.

Bridgwater, F. A. J., Morgan, R. E., Rowson, K. E. K. and Payling Wright, G.—(1955) *Brit. J. exp. Path.*, **36**, 447.

Eagle, H.—(1955) *Proc. Soc. exp. Biol., N.Y.*, **89**, 362.

Howard, J. G.—(1955) *Brit. J. exp. Path.*, **36**, 439.

Kaplan, A. S. and Lennox, E. S.—(1958) ' Abstracts of Communications. 7th International Congress for Microbiology,', Stockholm. Uppsala (Almqvist and Wiksells), p. 219.

Koch, P. K. and Olitzki, A. L.—(1946) *Exp. Med. Surg.*, **4**, 54.

Lawrence, J. C.—(1959) *Brit. J. exp. Path.*, **40**, 8.

Lennox, E. S. and Kaplan, A. S.—(1957) *Proc. Soc. exp. Biol., N.Y.*, **95**, 700.

Levaditi, C. and Muttermilch, S.—(1913) *C.R. Soc. Biol., Paris*, **74**, 379 and 614.

Olitzki, A. L., Bendersky, J. and Koch, P. K.—(1943) *J. Immunol.*, **46**, 71.

Penso, G. and Vicari, G.—(1957) ' Immunological Phenomena studied by the Tissue Culture Method '. Proc. 3rd International Meeting of Biological Standardization, Opatija, p. 11.—(1957*a*) *Rediconti dell'Accademia Nazionale dei Lincei*, serie 8, **22**, 772.—(1957*b*) *Rediconti dell'Istituto Superiore di Sanità*, **20**, 655–662.—(1957*c*) *Ibid.*, **20**, 1109.—(1959) *Ibid.*, **22**, 135 and 138.

Steabben, D.—(1943) *J. Hyg., Camb.*, **43**, 83.

Van Heyningen, W. E.—(1955) *Brit. J. exp. Path.*, **36**, 373 and 381.

Idem and Gladstone, G. P.—(1953) *Ibid.*, **34**, 202, 221 and 230.

THE AFFINITY OF THE RETICULO-ENDOTHELIAL SYSTEM FOR VARIOUS MODIFIED SERUM PROTEINS

G. J. THORBECKE, P. H. MAURER AND B. BENACERRAF

From the Departments of Pathology, New York University College of Medicine and University of Pittsburgh Medical School

Received for publication October 5, 1959

THE reticulo-endothelial (RE) cells of the liver and spleen phagocytose heat denatured proteins (Benacerraf, Biozzi, Halpern and Stiffel, 1956) or heavily iodinated proteins (Biozzi, Benacerraf, Stiffel, Halpern and Mouton, 1957) with great avidity. This phenomenon can be demonstrated by following the fate of ^{131}I labelled proteins after intravenous injection or, indirectly, by measuring the interference which these modified proteins exert on the rate of clearance of a standard dose of carbon particles from the blood by the RE system.

In the present study these observations are extended to the behaviour upon injection of other modified protein preparations derived from bovine serum albumin (BSA) in order to investigate the effect of physico-chemical changes and chemical modifications on the uptake of these proteins by the RE system.

Since considerable information is already available from the studies of Maurer, Sri Ram and Ehrenpreis (1957), Maurer and Sri Ram (1958) and Maurer (1959) on the correlation between antigenicity of BSA and modification of this protein by various chemical and physical means, these products have been used in these experiments.

MATERIALS AND METHODS

Animals.—The experiments were performed on male mice, weighing 25–35 g. All injections were given intravenously.

Protein preparations.—The chemically modified preparations (deaminated, acetylated, guanidinated, esterified) were prepared from Armour's crystalline BSA according to the previously published procedures (Maurer *et al.*, 1957; Sri Ram and Maurer, 1958 and 1959). The preparations modified by physicochemical treatments (including urea and ultraviolet (U.V.) treated) were prepared as outlined by Maurer (1959).

Sedimentation constants.—The S_{20w} of 1 per cent protein solutions in pH 7·4 phosphate buffer, $\mu = 0{\cdot}1$, were determined in a Spinco Model E ultracentrifuge run at a speed of 56,100 r.p.m.

Viscosity measurements.—Viscosity measurements were carried out at $25 \pm 0{\cdot}01°$ in Ostwald-Fenske viscometers as described by Ehrenpreis, Maurer and Sri Ram (1957).

Electrophoretic mobilities.—The mobilities were determined by paper electrophoresis in a veronal buffer of pH 8·6, $\mu = 0{\cdot}05$ as described by Ehrenpreis *et al.* (1957). Mobilities are reported relative to the value obtained for BSA.

Interference with phagocytosis of carbon particles.—Carbon preparation C11–1431a (manufactured by Gunther Wagner, Hanover) was used in these experiments. It was prepared as described by Biozzi, Benacerraf and Halpern (1953) and injected into mice in a dose of 16 mg./100 g. The carbon clearances were determined, according to the methods described, by drawing at regular time intervals 0·025 ml. blood samples from the retroorbital venous plexus with a calibrated glass pipette. previously rinsed with heparin. The blood samples were lysed in 2 ml. of 0·1 per cent sodium carbonate and the carbon concentration measured

spectrophotometrically at 675 mμ. The rate of clearance of carbon particles follows an exponential function of the time according to the equation $\frac{\log C_2 - \log C_1}{T_1 - T_2} = K$ in which C_1 represents the concentration of carbon in the blood at time, T_1, C_2 the concentration at time T_2. The rate constant K, called the phagocytic index, varies inversely with the injected dose of carbon in the dose range where the experiments were performed, illustrating the saturating effect of phagocytosed particles on the RE system. If, during the clearance of carbon particles, another colloidal suspension such as denatured protein, which is phagocytosed by the RE system with comparable avidity, is injected, a competitive effect on the clearance of carbon results and the rate of carbon clearance (K_1) is modified (K_2). The ratio of K_1 to K_2 measures the competitive effect on the carbon clearance and indicates the relative avidity with which various substrates are phagocytosed by the RE system as compared with carbon. After a sufficient number of blood samples had been obtained to measure the clearance rate K_1, the substance to be investigated was injected in the dose of 2·5 or 12·5 mg. per 100 g. body weight, and several more blood samples were withdrawn to allow measurement of a change in the clearance rate of carbon (K_2). Each protein preparation was investigated in 4 mice and mean values for K_1/K_2 are recorded in the Table.

Labelling of protein preparations with ^{131}I.—To 10 mg. of each protein preparation while kept at 0–5°, 0·15 ml. of a carbonate buffer (pH 10) and 0·28 ml. of a KI_3 solution containing 0·5 mc. of ^{131}I were added dropwise while shaking. The KI_3 solution contained 123 mg I_2 and 157 mg KI per 100 ml. distilled water. After an hour, the solutions were dialyzed against 0·85 per cent NaCl for 48 hr. The amount of iodine bound to protein was found to vary with the different preparations from 0·09 mg. to 0·41 mg. per 100 mg. of protein. The preparations which had been denatured by heat treatment seemed to bind less iodine (around 0·1 per cent) than the other preparations (around 0·3 per cent).

Clearance of ^{131}I *labelled proteins.*—Mice were injected i.v. with a standard dose of 5 mg. per 100 g. body weight of each ^{131}I labelled protein preparation. Samples of 0·05 ml. blood were drawn from the retro-orbital venous plexus at regular time intervals for a period of 30–90 min. after the injection, depending on the rate at which the protein disappeared from the circulation. The samples were placed on filter paper and mounted on glass slides. The radioactivity was measured with a Geiger-Müller counter and the results plotted as a percentage of radioactivity present immediately after the injection, against time. In order to study the fate of these modified proteins in the body, mice were injected i.v. with a standard dose of 1 mg. per 100 g. body weight of each labelled protein preparation and sacrificed 10 min. later. This short time interval was chosen to avoid metabolism of the phagocytosed protein and release of the label from the cells (Benacerraf, Halpern, Biozzi, Stiffel and Mouton, 1957; Biozzi, Halpern, Stiffel and Mouton, 1958). The γ-radioactivity of the spleen, liver, lung and kidneys was measured with a scintillation counter and expressed as a percentage of the injected radioactivity.

RESULTS

The fate of the various modified BSA preparations, injected intravenously, was determined by a study of the comparative speed of elimination from the blood, and of the distribution in various organs. The degree to which these preparations were able to interfere, by competition, with the phagocytosis of carbon particles by the RE system was studied and correlated with the extent to which they were removed by the liver.

These findings were further correlated with various physical constants, such as sedimentation constants, relative viscosity and electrophoretic mobility (Table I, Fig. 1 and 2).

Substances, which are phagocytosed with great avidity by the RE system, are characterized by: a relatively fast disappearance rate from the blood, a high degree of liver uptake and a marked competitive effect on phagocytosis of carbon particles *in vivo*. Among the derivatives of BSA showing this behaviour we can list the following: U.V. denatured, deaminated-P, N-acetyl BSA, urea treated

TABLE

Protein prep. of BSA	K_1/K_2*	Organ distribution†				Electroph. mobility	Relative viscosity 1 per cent pH 7·5	Sedim. constants S_{20} w
		Liver	Spleen	Kidneys	Lung			
Guanidinated (1)	1·4	19	0	6·5	5	1·05	1·051	92 per cent : 4·1 8 per cent : 6·3
Deam. SIII (2)	1·3	10·5	0	9·5	2	—	1·041	100 per cent : 3·3
Deam. P (3)	2·6	76	4	17	0	80 per cent : 1·34	1·100	65 per cent : 3·8 35 per cent : 5·3
n-acetylated (4)	5·0	52	1·9	6·9	2·8	75 per cent : 1·71	1·081	85 per cent : 3·1 15 per cent : 2·1
Heat denatured guanidinated (5)	5·0	76	4	17	0	70 per cent : 1·04	—	70 per cent : 18·8 25 per cent : 4·3 5 per cent : 9·0
Irreversibly urea denatured (6)	1·6	29	2	28	0	—	1·065	90 per cent : 2·0 10 per cent : 8·4
Urea denatured heat denatured (7)	2·7	67	2	13	0	—	1·182	37 per cent : 3·4 34 per cent : 1·4 29 per cent : 9·3
Ultraviolet denatured(8)	2·8	74	0	6	3	—	1·025	83 per cent : 5·9 10 per cent : 2·8
Alkali denatured (9)	1·9	29	2	40	0	—	1·054	90 per cent : 2·0 10 per cent : 10·0
Acid denatured (10)	1·2	10	2	45	2	—	1·035	100 per cent : 1·5
Methylated‡ (11)	—	74	2·4	2	2·7	—	1·35	77 per cent : 3·6 23 per cent : 9·6
Normal (12)	1	—	—	—	—	1·00	1·040	92 per cent : 4·0 8 per cent : 5·8
Normal‡ (13)	—	—	—	—	—	—	1·29	85 per cent : 3·7 7·5 per cent : 5·4 7·5 per cent : 2·4

Competition with carbon clearance (K_1/K) and percentage uptake by the liver as correlated with physicochemical properties of various modified BSA preparations.

* Dose of carbon for clearance : 16 mg./100 g. ; dose of protein 1–5 : 12·5 mg./100 g. ; 6, 7, 9, 10 : 10 mg./100 g. 8 : 6 mg./100 g.

† Determined at 10 min. after i.v. injection of 1 mg./100 g.

‡ Physicochemical data obtained in 0·02 N HCl as solvent.

heat denatured, and heat denatured guanidinated. All these preparations show about 70 per cent of liver clearance, with a great degree of efficiency, and considerable interference with the phagocytosis of carbon : $K_1/K_2 > 2{\cdot}5$ in all cases. These preparations, however, are not homogeneous, since the rates of clearance from the blood do not follow an exponential function of time as they would with substances which are homogeneously phagocytosed by the RE system. This inhomogeneity is also apparent from the data on sedimentation constants.

With the majority of these substances there is a correlation between the degree of aggregation and the rate at which they are removed by the RE system. Among these, heat denatured guanidinated BSA has a $S_{20} = 18{\cdot}8$ for 70 per cent of the material, urea denatured heat denatured a $S_{20} = 9{\cdot}3$ for 29 per cent, and U.V. denatured a $S_{20} = 5{\cdot}9$ for 83 per cent. Other preparations such as deaminated-P preparation which has a $S_{20} = 3{\cdot}8$ for 65 per cent and N-acetylated BSA which has a S_{20} of 3·1 for 85 per cent and of 2·1 for 15 per cent, are also phagocytosed by the Kupffer cells of the liver without evidence of considerable aggregation accompanying the denaturation of these products. The extent of aggregation and/or denaturation is also indicated by changes in relative viscosity and may account for the large competitive effect on carbon clearance of heat denatured

guanidinated and acetylated BSA (both $K_1/K_2 = 5$). The former product shows considerable aggregation, while the latter shows a marked increase in negative net charge.

Other BSA derivatives show a rapid clearance from the blood, and yet, small or negligible interference with phagocytosis of carbon and uptake by the liver.

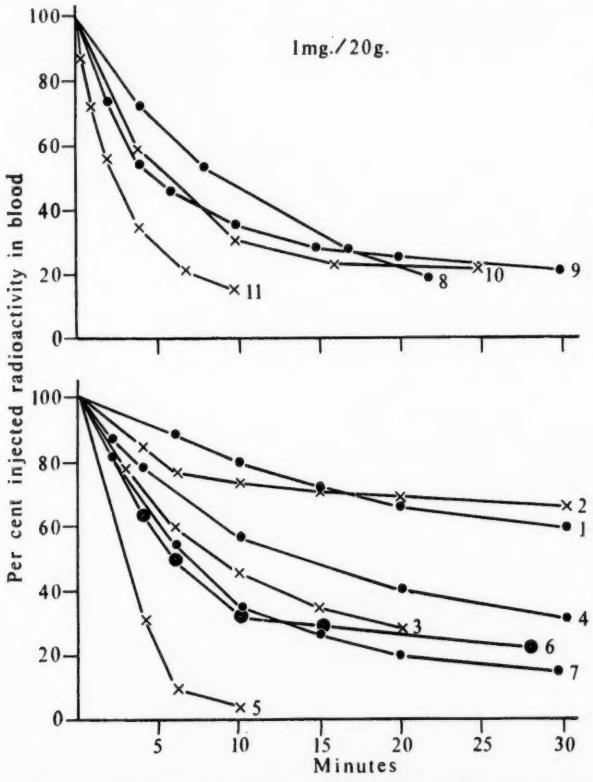


FIG. 1.—Rate of disappearance of intravenously injected ^{131}I labelled modified BSA preparations (5 mg./100 g.) from the blood of mice. The numbers refer each to a different preparation: (1) guanidinated, (2) deaminated SIII, (3) deaminated P, (4) *n*-acetylated, (5) heat denatured guanidinated, (6) irreversibly urea denatured, (7) urea denatured heat denatured, (8) ultraviolet denatured, (9) alkali denatured, (10) acid denatured, (11) methylated.

Among these, we find acid denatured, alkali denatured and urea denatured BSA. Alkali denatured and urea denatured BSA, which both have 10 per cent of an aggregated component, show K_1/K_2 ratios of 1·0 and 1·6 respectively, and 29 per cent clearance by the liver only. However, it is clear from the analysis of the organs, that the kidneys are largely responsible for the rapid clearance of these three products which have been hydrolyzed during the preparation, as evidenced by sedimentation constants considerably smaller than the ones for normal BSA

(acid denatured 100 per cent: 1·5; alkali denatured 90 per cent: 2·0; urea denatured 90 per cent: 2·0).

Guanidinated BSA and the SIII fraction of deaminated BSA which have been changed very little by chemical treatment are not eliminated very rapidly from

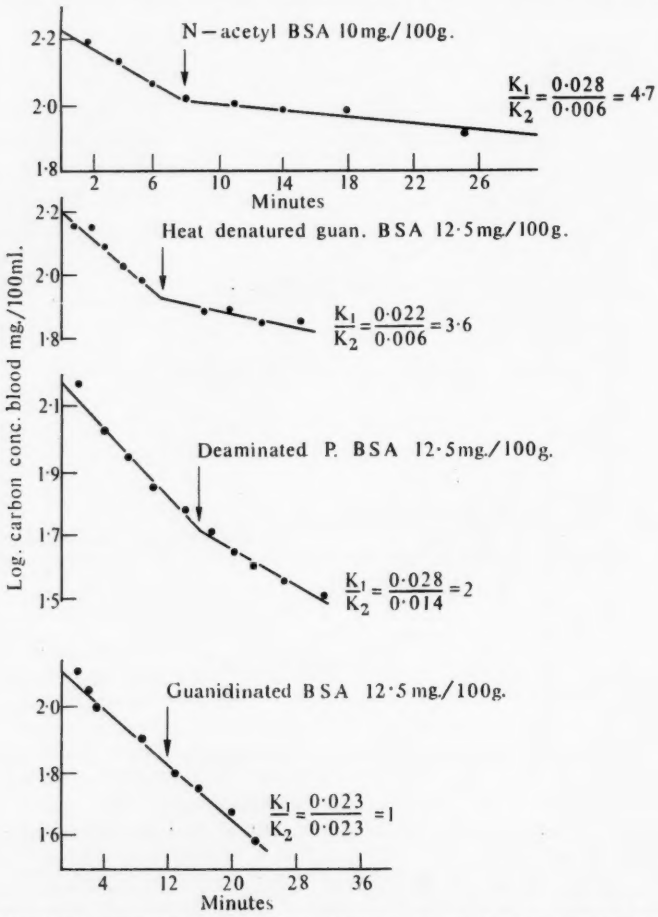
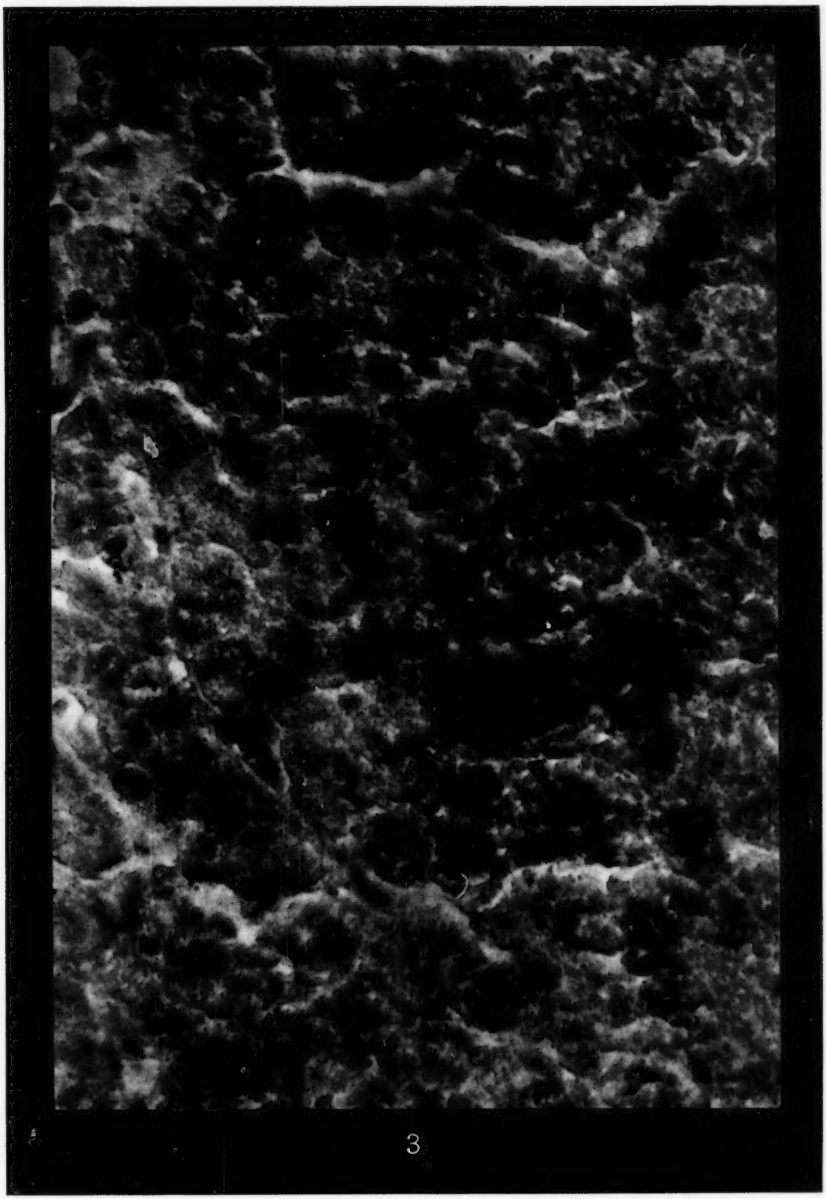


FIG. 2.—Graphical representation of carbon clearance curves showing varying degrees of interference by the injection of modified BSA preparations. Arrows indicate time of protein injections.

EXPLANATION OF PLATE.

FIG. 3.—Autoradiography of liver section of a mouse injected with ^{131}I labelled *n*-acetylated BSA, showing localization of radioactivity in Kupffer cells. Autoradiograph and photograph were prepared by Dr. R. T. McCluskey, Department of Pathology, New York University College of Medicine.



3

Thorbecke, Maurer and Benacerraf.

the circulation and, as expected, are not recovered, in any significant amount, in the organs when the animals are sacrificed 10 min. after the injection. They also do not show any appreciable effect on the rate of carbon clearance since any ratio $K_1/K_2 < 1{\cdot}5$ must be considered to be of questionable significance and within the limits of error of this technique.

All the modified proteins studied heretofore are negatively charged at the pH of blood. When a positively charged derivative of BSA is injected, such as esterified (methylated) BSA, the preparation is cleared rapidly from the blood and 74 per cent recovered in the liver after 10 min. If methylated BSA is injected in the course of clearance of carbon from the blood, a sharp decrease in carbon concentration is observed. The behaviour of this preparation *in vivo* can be explained by the reaction which it shows with serum *in vitro*. This positively charged colloid precipitates rapidly with the negatively charged serum proteins (Sri Ram and Maurer, 1959). It reacts similarly with negatively charged carbon particles. Because of this, positively charged soluble proteins are precipitated in the blood. It is impossible, therefore, to investigate by our techniques whether such proteins can be phagocytosed by the RE system from a colloidal solution.

All modified BSA preparations were less antigenic than native BSA (Maurer *et al.*, 1957 ; Sri Ram and Maurer, 1957 and 1958 ; Maurer and Sri Ram, 1958). Most comparable to native BSA, in this respect as well, were guanidinated BSA and the SIII fraction of deaminated BSA. A correlation between the tendency of these substances to localize in the RE system of the liver and their degree of antigenicity was not found. The only preparation which was not antigenic in the rabbit when injected as an alum precipitate was acid denatured BSA.

DISCUSSION

The term denaturation refers to several physicochemical changes which can occur during modification of a native protein (Putnam, 1953). Upon " denaturation " of a protein various masked polar groups, such as —SH, tyrosyl and amino groups may become exposed because of " unfolding " and/or expansion of the molecules. Aggregation as a result of reassociation of exposed groups is a secondary phenomenon dependent on the electrokinetic potential of the denatured protein particles. The solubility of denatured proteins is always less than that of normal globular proteins, particularly at the isoelectric point.

Among the several changes that various treatments induce in normal BSA, and that modify the fate of these products upon intravenous injection, a change of molecular size seems to be of major importance. The proteins that have been subjected to partial hydrolysis, such as acid, alkali or urea treated BSA showed a rapid clearance by the kidney, while the ones that were aggregated were phagocytosed by the liver with great avidity. However, in addition to aggregation of the molecules a change in the negative surface charge of the molecules enhanced the affinity of these substances for the RE system as well (deaminated-P fraction, N-acetylated BSA).

What information may one derive from these studies about the factors that are responsible for the greater affinity of some of these products for the RE system? Substances to be phagocytosed have to be adsorbed to the RE cell membranes for a sufficient length of time to initiate the process which transports them inside the cell. The criteria for specific adsorption onto the cell surface

may thus be of major importance in determining the degree of phagocytosis. It seems that the large molecular size of the denatured proteins, irrespective of the method of denaturation, greatly enhances their affinity for the RE cells. On the other hand, such a large molecular size is not the only property which renders proteins capable of being phagocytosed, as some preparations which were not aggregated were avidly phagocytosed. Other factors, such as surface charge and diminished solubility at the pH of blood plasma, may therefore also be of great importance in determining adsorption of these products onto phagocytic cells.

SUMMARY

The fate of modified BSA preparations was studied in mice after intravenous injection. The rate of disappearance from the blood of lightly ^{131}I labelled protein preparations and their distribution in various organs (liver, spleen, kidneys, lung) at 10 min. after an intravenous injection were studied. Further information about the affinity of these substances for the RE system was obtained by studying the interference exerted by the modified proteins on the clearance of a standard dose of carbon particles.

An attempt was made to correlate these results with the physicochemical properties of the preparations, such as relative viscosity, electrophoretic mobility and sedimentation constants.

It was found that partially hydrolyzed protein preparations like acid denatured, alkali denatured and urea denatured BSA, were rapidly cleared from the blood by the kidney, while substances which had not been changed appreciably physicochemically, such as guanidinated and lightly deaminated BSA behaved similarly to normal BSA.

The RE system was shown to remove selectively highly aggregated protein preparations such as heat denatured guanidinated and urea denatured heat denatured BSA, and also proteins which were not aggregated much upon physicochemical treatment, but which had significantly changed negative surface charge, like strongly deaminated and *n*-acetylated BSA.

No correlation was found between uptake by the RE system of the liver and the degree of antigenicity of the protein preparations.

The factors influencing the affinity of modified proteins for the RE system are discussed.

These studies were supported by grant Nos. E 1268 and E 2094 from the National Institutes of Allergy and Infectious Diseases (United States Public Health Service).

REFERENCES

BENACERRAF, B., BIOZZI, G., HALPERN, B. N. AND STIFFEL, C.—(1956) RES Bulletin 2, 19.

Idem, HALPERN, B. N., BIOZZI, G., STIFFEL, C. AND MOUTON, D.—(1957) *Brit. J. exp. Path.*, **38**, 35.

BIOZZI, G., BENACERRAF, B. AND HALPERN, B. N.—(1953) *Ibid.*, **34**, 441.

Idem, BENACERRAE, B., STIFFEL, C., HALPERN, B. N. AND MOUTON, D.—(1957) *Ann. Inst. Pasteur*, **92**, 89.

Idem, HALPERN, B. N., STIFFEL, C. AND MOUTON, D.—(1958) *Brit. J. exp. Path.*, **39**, 510.

EHRENPREIS, S., MAURER, P. H. AND SRI RAM, J.—(1957) *Arch. Biochem. Biophys.*, **67**, 178.

MAURER, P. H.—(1959) *Ibid.*, **79**, 13.

Idem AND SRI RAM, J.—(1958) In XIV Annual Protein Conference, Rutgers Univ. Press, p. 56.

Iidem AND EHRENPREIS, S.—(1957) *Arch. Biochem. Biophys.*, **67**, 196.

PUTNAM, F. W.—(1953) In ' The Proteins '. New York. (Acad. Press), v. I B, p. 807.

SRI RAM, J. AND MAURER, P. H.—(1957) *Arch. Biochem. Biophys.*, **72**, 119.—(1958) *Ibid.*, **74**, 119.—(1959) *Ibid.*, **83**, 223.

ORAL AND DENTAL LESIONS IN VITAMIN B_6 DEFICIENT RHESUS MONKEYS*

C. C. BERDJIS,† L. D. GREENBERG, J. F. RINEHART‡ AND G. FITZGERALD

From the Department of Pathology, University of California School of Medicine and the Section of Oral Roentgenology, College of Dentistry, University of California, San Francisco

Received for publication October 16, 1959

DURING studies of the biochemical and pathological changes in rhesus monkeys subjected to vitamin B_6 deprivation, the teeth of animals that had been exposed to rather prolonged deficiency underwent marked deterioration. As a result of this observation systematic study was conducted on the teeth and peridontal tissues of control and vitamin B_6 deficient monkeys, and also of animals that had been subjected to deficiences of other vitamins. The present paper is concerned only with vitamin B_6 deficiency. Some of this work has been published previously (Rinehart and Greenberg, 1956). To date, the teeth of 81 animals have been examined, of which 29 had been subjected to varying degrees and periods of pyridoxine deficiency. Only brief reports of oral lesions in vitamin B_6 deficient animals have previously been presented by Birch, Gyorgy and Harris (1935) and Afonsky (1955).

The advantages of the monkey as a subject for experimental dental caries research have been pointed out by Johansen and Keyes (1955) and are briefly mentioned here. The dental formula of the monkey is the same as that of man and their teeth compare favourably in size, shape, and composition with those of man. Other factors which favour the use of the monkey for research on dental caries are that it possesses, similar to the human, a set of deciduous teeth, and that the carious lesions in the monkey resemble very closely the lesions of human teeth both macroscopically and microscopically.

Previous studies in monkeys by others (Schultz, 1935, Shaw, Elvehjem and Phillips, 1945, and Knap, 1941) have revealed a low incidence and low susceptibility of the rhesus monkey to dental caries. Shaw *et al.* (1945) have reported a much higher incidence of caries in rhesus monkeys maintained on purified rations. However, their results represent pooled or composite data on monkeys maintained on nutritionally adequate as well as vitamin deficient diets.

METHODS AND MATERIALS

The animals used in these experiments were young, immature, predominantly male, rhesus monkeys, weighing between 1·8 and 2·5 kg. at the time of arrival at the laboratory.

* Supported by grants from the following sources : A-645 USPH ; Merck and Co., Inc., Rahway, New Jersey ; National Vitamin Foundation, Inc., New York ; Nutrition Foundation, Inc., New York ; and The University of California.

† Present address : Lt.-Col., M.C., U.S. Army Medical Unit (Walter Reed Army Medical Center), Fort Detrick, Maryland.

‡ Deceased.

They were estimated to be 1–1½ yr. of age. With few exceptions, the care, diet and vitamin supplements used were essentially those described by Rinehart and Greenberg (1956). Deficient animals received the same diet as control animals except for the omission of the vitamin under investigation.

The skulls of animals which had died or had been killed were preserved in 10 per cent formalin. In some cases only one jaw was saved. The incidence of caries was computed from an examination of the surface lesions and checked in most cases by radiological examination. After cleaning and separation of the jaws, photographs were taken of the frontal, occlusal and lateral views. Radiographs were first taken of the anterior teeth, and then each jaw was sawed vertically in the mid-line, and each half was radiographed laterally. For histological study, mediodistal, labio-lingual and sagittal sections were made of the incisors, premolars, and molars. They were decalcified, embedded in paraffin or celloidin and stained with haematoxylin and eosin, Mallory, Masson or Gomori's trichrome stain, and by periodic acid Schiff (PAS) or the colloidal iron method for mucopolysaccharides.

In a few instances complete examination was made of the teeth of young monkeys under pentobarbital anaesthesia.

RESULTS

Macroscopic examination

Teeth.—The teeth of vitamin B_6 deficient animals were reduced in size and altered in shape in many cases, such changes being particularly prominent in the incisors. Frequently the incisors were studded with opaque or dirty enamel. In addition, certain teeth showed brownish discolouration and mottled enamel; this was more apparent in deficient than in the control animals. In some instances the teeth were dirty grey or bright pearly grey in colour. The anterior teeth, particularly the incisors, were the most affected by pyridoxine deficiency. They were frequently irregular, atrophic and considerably shortened and hypoplastic. Deformed, shortened, and stunted teeth have occasionally been present among other teeth. The failure of normal development of the canine teeth was particularly striking in some instances (Fig. 1, *a* and *b*).

In some cases the teeth seemed to be soft and exhibited marked attrition. Various degrees of attrition were frequently encountered in almost all categories of teeth, including premature attrition of the deciduous teeth (Fig. 1, *a* and *b*).

Another significant change in the teeth of pyridoxine deficient monkeys was decalcification. Teeth with various degrees of decalcification or attrition were not uncommon. Examples of these are shown in Fig. 2. The incisors frequently show loss of enamel with exposure of the dentine at the labial and lingual surfaces. The other classes of teeth were less frequently involved.

Caries.—In agreement with the observations of others (Schultz, 1935; Shaw *et al.*, 1945) carious lesions of the deciduous teeth were rarely observed in either control or vitamin deficient monkeys. Deficiency of vitamin B_6 for periods of a year or less had little or no effect on the incidence of caries. At the termination of these experiments the animals were generally in possession of most of their deciduous teeth and caries were of infrequent occurrence regardless of whether or not the monkeys had received an adequate intake of pyridoxine.

The frequent occurrence of caries became evident in monkeys that had been maintained on deficient or suboptimal intakes of vitamin B_6 for periods of 16 months or longer. At this time the animals were estimated to be at least 3 yr. of age and the second dentition had begun or was complete.

A summary of the incidence of caries in the teeth of control and vitamin B_6 deficient monkeys is presented in Table I. The animals have been grouped in

TABLE I.—*Carious Teeth in Vitamin B_6 Deficient and Control Monkeys*

Experimental period	Number of animals	Total teeth examined	Total carious teeth	Carious teeth (per cent)
Controls ;				
<3 yrs.*	11	244	17	7
>3 yrs.†	10	304	44	14
Total	21	548	61	
Deficient ;				
7–15 months	14	246	25	10
16–56 months	14	288	115	40
Total	28	534	140	

* 5–22 months.
† 4¼–7⅓ yrs.

EXPLANATION OF PLATES

FIG. 1.—Frontal view of the upper jaw of a control (*a*) and of a vitamin B_6 deficient monkey (*b*), both adults of the same age (5 yr. approx.). Points of particular significance in (*b*) are: Mal-alignment and the presence of small, stunted anterior teeth ; swollen and hyperplastic gingivae ; acute gingivitis ; defects in the development and incomplete eruption of the canines.

FIG. 2.—Frontal view of an adult pyridoxine deficient monkey (compare with Fig. 1*a*) exhibiting marked decalcification of the teeth.

FIG. 3.—Occlusal view of the lower jaw of a control (*a*) and of a pyridoxine deficient (*b*) monkey of same age (5 yr. approx.). Caries of all class of teeth, marked decalcification and attrition of the anterior teeth, fissuring and ulceration of the gingivae are conspicuous in (*b*). The canines are again incompletely erupted.

FIG. 4.—Frontal view of the lower jaws as shown in Fig. 3 ; (*a*) control, (*b*) pyridoxine deficient.

FIG. 5.—Mesiodistal section of the anterior teeth in a young adult pyridoxine deficient monkey. Downgrowth of the epithelium, fissuring, ulceration and calculus are visible. Resorptive atrophy of the bone and atrophy of the pulp are also shown in this section. H. and E. ×24.

FIG. 6.—Mesiodistal section of the lower jaw in an adult pyridoxine deficient monkey showing the crevicular epithelium with a large ulceration and superimposed calculus. Fragmented epithelium is surrounded by a chronic inflammatory reaction. H. and E. ×70.

FIG. 7.—Labio-lingual section of a lower lateral incisor in a pyridoxine deficient monkey showing advanced attrition and marked proliferation of the gingivae. This incisor shows extensive odontal resorption everywhere with proliferation of the periodontal tissues into resorption areas. There is also marked resorption of the labial and lingual plate of bone. H. and E. ×24.

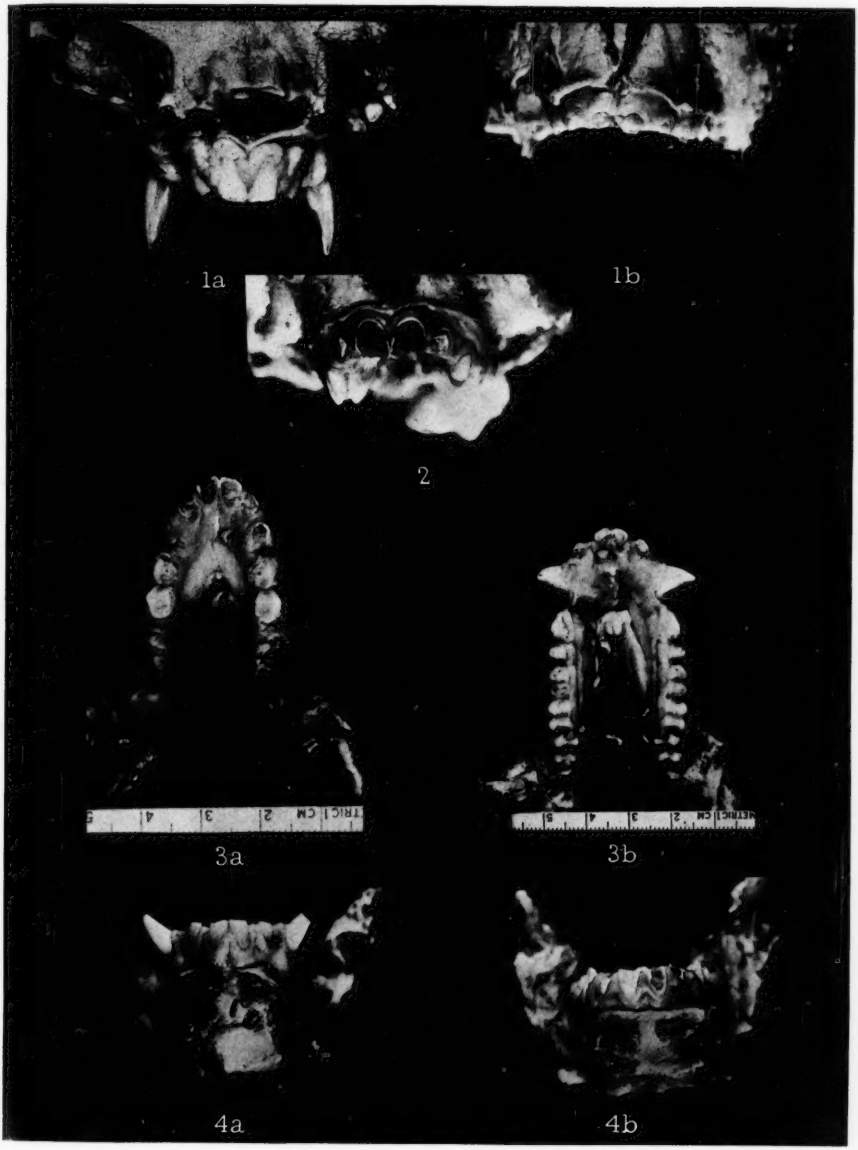
FIG. 8.—Higher magnification of Fig. 7 showing a relatively large " lacunar " resorption of the incisor filled with a fibrous and granulomatous mass of periodontal tissues containing numerous multinucleated giant cells (cementoclasta). No hypercementosis is visible on the edge of this " lacunar " area. This lesion is similar to human external or idiopathic odontal resorption. H. and E. ×85.

FIG. 9.—Mesiodistal section of the lower right molars and premolar in a vitamin B_6 deficient monkey showing progressive atrophy of the interdental papillae and of the alveolar bone, and hypercementosis. H. and E. ×24.

FIG. 10.—Odontal resorption with hypercementosis in a root of an incisor and extensive bone destruction consecutive to the chronic osteomyelitis. H. and E. ×60.

FIG. 11.—A fragment of the root of an incisor surrounded by inflammatory cells and fibrous tissue which are intermingled with a few epithelial fragments. H. and E. ×60.

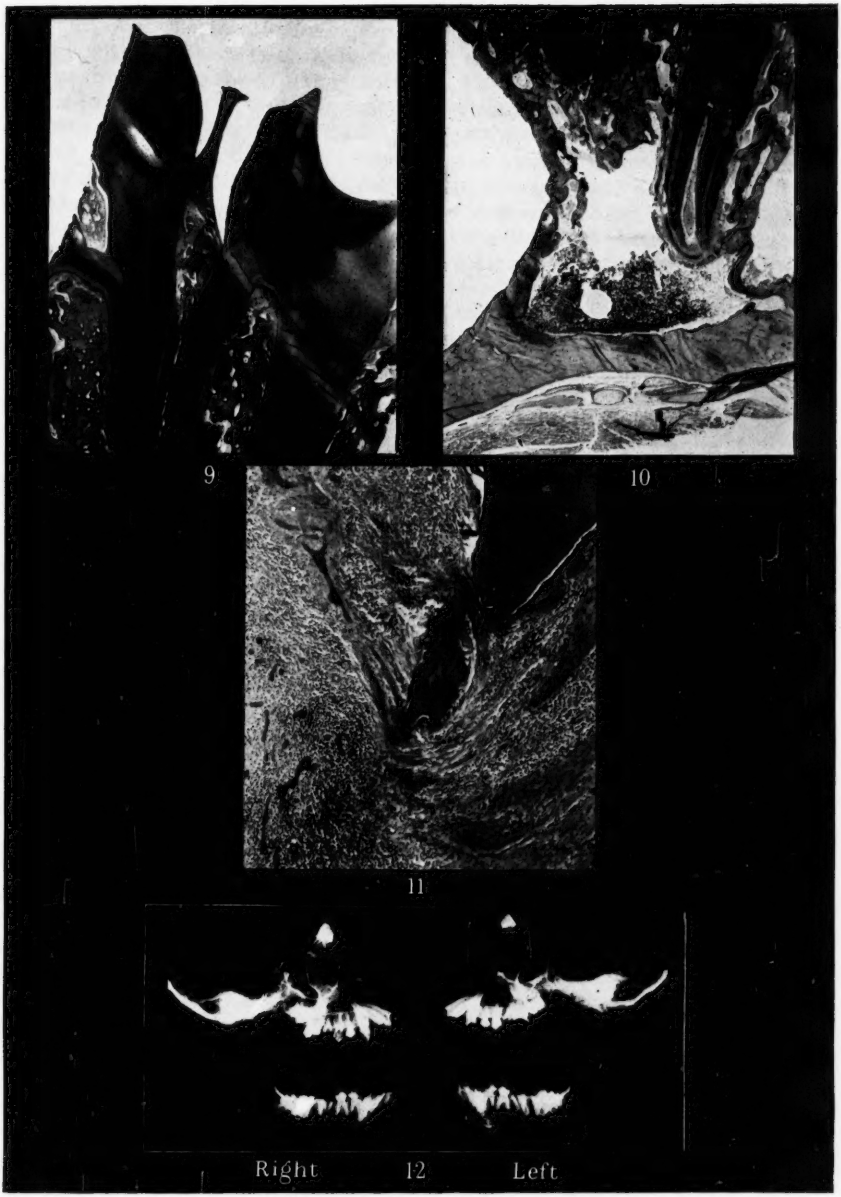
FIG. 12.—Radiograph of the jaws of a pyridoxine deficient monkey showing attrition, caries of all classes of teeth, periodontal and apical abscesses, periapical calcification, odontal resorption interdental bone resorption and osteoporosis of the bone.


1a
1b
2
3a
3b
4a
4b

5 6

7 8

Berdjis, Greenberg, Rinehart and Fitzgerald.



Berdjis, Greenberg, Rinehart and Fitzgerald.

accordance with the duration of exposure to their respective diets. In the controls it is evident that there is some increase in the incidence of caries with respect to age or duration on the experimental diet. The average increase in the incidence of caries in the group of control animals maintained on the diet for 3 yr. or longer amounts to only 7 per cent or a 2-fold increase over that of monkeys fed the same diet for periods of less than 3 yr. However, the marked increase in the incidence of caries of monkeys subjected to prolonged or intermittent vitamin B_6 deficiency is striking. Whereas the incidence of caries was only 10 per cent in the group of monkeys which had been maintained on the deficient diet for 7–15 months, the incidence increased to 40 per cent or 4-fold in the group of monkeys which had been subjected to the deficient diet for periods of 16–56 months.

In control animals the percentage of total teeth affected with caries was slightly higher in the mandible (55 per cent) than it was in the maxillae (45 per cent). The reverse was true in the case of vitamin B_6 deficient monkeys (maxillae 53 per cent ; mandible 47 per cent).

The distribution of the carious lesions in vitamin B_6 deprived monkeys according to class or type of teeth is shown in Table II. The results show quite clearly that there is marked increase in caries in all classes of teeth of monkeys subjected to the more protracted deficiency. The increase in the caries incidence is relatively and comparatively greatest in the canines (C), incisors (I), and premolars (P), and less in the molars (M). (The ratio for the former is roughly 4 : 1 and for the latter 2 : 1.) The results summarized in Table III show the first and second molars have about 25 per cent carious lesions, whereas the other teeth are seldom affected (Table III). Therefore, in order of decreasing susceptibility or predilection the teeth fall into the following sequence : M_1, M_2, P_1, P_2, I_1, and C (Table III).

TABLE II.—*Distribution of Caries in Vitamin B_6 Deficient and Control Monkeys*

	Controls			Deficient			
		5·3–12 months	39–87 months	7–15 months		16–56 months	
Class of teeth	Total No.* teeth	Carious teeth (per cent)		Total No. teeth	Carious teeth (per cent)	Total No. teeth	Carious teeth (per cent)
Incisor (I)	104	0	9	80	7·5	80	35
Canine (C)	52	2·5	5	40	3	40	25
Premolar	104	5	7	80	11	80	36
Molar	108	20	27	46	20	88	53

* Combined total for both control groups.

TABLE III.—*Susceptibility of Various Classes of Teeth of the Pyridoxine-Deficient Monkey to Caries*

Class of teeth	Total No. teeth	Total carious teeth	Carious teeth (per cent)
I_1	84	16	19
I_2	84	19	22
C	84	12	14
P_1	84	21	25
P_2	84	17	20
M_1	84	42	50
M_2	40	17	40

Gingivae.—The gingivae also show the effects of vitamin B_6 deficiency in the form of acute and chronic gingivitis. In the controls the gum margin was firm, pink and solidly attached to the teeth; whereas in the deficient animals with acute gingivitis the gums were swollen, hypertrophied, bright pink or bright red with a tendency to fill the interdental space (lobulation of interdental papillae). In some cases small superficial ulcerations were visible not only along gum margins but also on the floor of the mouth or the palate mucosae (Fig. 3, *a* and *b*). Varying amounts of calculus were present in this stage of acute gingivitis. Shallow ulcers were observed occasionally and when present were usually located in the anterior part of the oral cavity adjacent to the incisors.

In the chronic stage, the gingivae were pale, firm and fibrous, or greyish-red to greyish-dirty when fissures and ulcers were present. Loss of uniform colour, partial thickening, partial atrophy of the gingiva, roughness, discolouration of the interdental papillae, with or without ulceration, were observed after long periods of pyridoxine deficiency. In some cases the chronic gingivitis was complicated by recession of the gingivae and formation of shallow pockets. The latter were associated with varying amounts of calculus surrounded by a purulent exudate (Fig. 4, *a* and *b*).

Tongue.—The lingual lesions in pyridoxine deficient monkeys were essentially those of atrophic glossitis. The surface of the tongue was somewhat furrowed, particularly in the anterior third and was pink or bright red in colour. A few small erosions and moist surface areas were seen in the early stages of the deficiency. The gross sections of tongue in various places showed marked atrophy of the epithelium, particularly in the anterior part, and some pale fibrous tissue between the atrophied muscle bundles.

Microscopic examination

The jaws of the pyridoxine deficient monkeys showed in general a progressive atrophy of bone and soft tissues which was frequently associated with inflammatory reaction of all the tissues surrounding the teeth. The teeth and the alveolar bone, including maxillary or mandibular bone were also subject to involvement by this progressive atrophy.

Characteristic changes occurred in the periodontal tissues (Fig. 5, 6, and 7). The interdental papillae showed exfoliation, marked atrophy or extensive destruction, which was most severe in long-term deficiency. The crevicular epithelium showed marked hyperplasia in some cases, or atrophy and fragmentation in others. The latter was occasionally the site of necrosis or ulceration. Superficial or deep ulcers were associated with calculi. Isolated papillary formations with frayed epithelial fragments, surrounded or mingled with round cell infiltration were not infrequent. In addition, degenerative epithelium with secondary inflammation was also seen in some preparations. On the gum margins down-growth of the epithelium of the alveolar architecture was frequently observed. Fissuring and cracking of the epithelium occurred commonly. From these fissures inflammatory cells extended into all periodontal tissues, leading to ulcerative gingivitis or abscesses under certain circumstances. On the surface of the gum margin, particularly in the early stages of pyridoxine deficiency, cornified or parakeratotic layers with desquamated epithelial cells were usually present. Likewise, the subepithelial tissue underwent oedematous and/or hyperaemic transformation with varying degrees of inflammatory reaction.

Apart from various stages of gingivitis and periodontal destruction, the teeth showed an unusual and particularly significant change, *i.e.*, dental resorption. The dental resorption may affect a relatively small area of a tooth or become generalized and extensive. By analogy with bone resorption, the former is designated " lacunar resorption " and the latter " generalized odontal resorption ". Many types of resorption including root resorption, lateral or apical resorption have been seen in the teeth of the pyridoxine deficient monkeys. The adjacent tissues, namely, pulp, enamel, cementum and periodontal membrane may be normal in appearance or may show more or less marked changes. Various degrees of pulpitis, with or without abscesses, hypercementosis, partial destruction of periodontal membrane, interradicular or periapical abscesses may accompany the dental resorption.

Fig. 8 shows a relatively large " lacunar resorption " with extensive destruction of enamel and multiple small foci of dental resorption in an incisor. The foci of dental resorption in pyridoxine deficiency are composed of a granular tissue rich in fibrous material and round cells with or without osteoclasts on the periphery and some capillaries intermingled with a few destroyed particles of dentine. No new dentine or membranous capsule has been seen in any area exhibiting this kind of a resorption. Root fractures or dental fragments have been noted in a few cases as a result of dental resorption.

Bone changes were characterized by a slight resorption of interdental and marginal alveolar bone including periapical bone, with fibrosis and some osteoclasts around the rarefied trabeculae. Osteoporosis was noted in 2 cases. A condition resembling osteoporosis was not infrequent because of the enlargement of marrow spaces and proliferation of peridental fibrous connective tissue ; the latter process was considered to be a reaction to chronic osteomyelitis. Resorption of alveolar and supporting bone are evident in Fig. 5, 7, 9, 10, and 11.

Most of the dental lesions described were evident in radiographs, an example of which is shown in Fig. 12. The latter represents a jaw of a vitamin B_6 deficient monkey in which the following are evident : caries of all classes of teeth, periodontal and apical abscesses, periapical calcification, odontal resorption, interdental bone resorption and osteoporosis of the bone.

DISCUSSION

The marked increase in the incidence of caries in prolonged deficiency of vitamin B_6 appears exceedingly impressive when one considers that our own and previous studies by others (Schultz, 1935 ; Shaw *et al.*, 1945 : Knap, 1941) have revealed a low incidence and low susceptibility of the rhesus monkey to dental caries.

The relatively high incidence of caries in the monkeys maintained on the pyridoxine-deficient diet for 16 months or longer and the infrequent occurrence of caries in the deciduous teeth of the rhesus monkey even when subjected to vitamin B_6 deprivation would be consistent with the concept that pyridoxine deficiency exerts a markedly deleterious effect on the course of development of the permanent teeth. This is further supported by the failure of normal development of the canine teeth and by the presence of deformed, shortened, and stunted teeth in the pyridoxine deprived monkey. Muschett (1956) has also observed mal-alignment of the teeth in vitamin B_6 deficient monkeys.

Whether vitamin B_6 deficiency is related to the occurrence of caries in humans is not known. It is possible that mild deficiency or suboptimal intakes of the vitamin operating over protracted periods of time during early childhood might be an aetiological factor in the development of carious teeth in humans. It has been estimated that the average daily intake of pyridoxine by man in the United States is approximately 1·5 mg. However, some doubt exists concerning the adequacy of this amount of vitamin B_6 for optimum metabolism. Rinehart and Greenberg (1956) have shown that the monkey requires approximately 50 μg. of pyridoxine (62 μg. of the hydrochloride) per kilogram body weight per day for optimal growth. If this datum is applicable to man, an individual weighing 70 kg. would require 3·5 mg., or 2·3 times the average daily intake. Schroeder (1955) has arrived at a similar opinion concerning inadequacy of the average intake of pyridoxine in the diet.

Since the publication of our preliminary report (Rinehart and Greenberg, 1956) several articles dealing with vitamin B_6 deficiency and caries have appeared. Steinman and Hardinge (1958) have reported that rats maintained on an average American diet had greater incidence of caries than rats fed the same diet supplemented with pyridoxine. Strean, Gilfillan and Emerson (1956) have described greater loss of tooth structure in hamsters maintained on a cariogenic diet and deprived of vitamin B_6 than in control hamsters receiving the same diet supplemented with pyridoxine. Strean, Bell, Gilfillan, Emerson and Howe (1958) reported there was a significant reduction of dental caries in the mandibular molars of hamsters which had received pyridoxine in a concentration of 10 parts per million as compared to a group which had received the vitamin in a concentration of 0·5 parts per million. They further reported a 40 per cent reduction in dental caries of a group of children receiving pyridoxine 3 mg. 3 times a day for a period of a year as compared with a similar group receiving placebos. They attributed the cariogenic effect of vitamin B_6 deficiency to an alteration of the oral flora.

SUMMARY

Rhesus monkeys maintained on diets deficient or suboptimal in pyridoxine for 16 months or longer exhibit a marked increase in the incidence of dental caries. Deficiency of vitamin B_6 was observed to have little or no effect on the incidence of caries in animals maintained on the deficient diet for periods of a year or less. In addition to the increased incidence of dental caries, the deficiency resulted in deformed, shortened, mal-aligned teeth and failure of normal development of the canine teeth. Erosion and decalcification of the teeth were also prominent and significant alterations resulting from the deficiency. Pyridoxine deficiency appears to exert its deleterious effects on the permanent teeth during the course of their development.

Atrophic glossitis and acute and chronic gingivitis were observed to occur in monkeys deprived of vitamin B_6. The possible relationship of vitamin B_6 deficiency to human caries is discussed.

REFERENCES

Afonsky, D.—(1955) *Oral Surg.*, **8**, 438.

Birch, T. W., Gyorgy, P. and Harris, L. J.—(1935) *Biochem. J.*, **29**, 2830.

Johansen, E. and Keyes, P. H.—(1955) in 'Advances in Experimental Caries Research'. Ed. Soggnaes, R. F., (A.A.A.S.) Washington p. 10.

KNAP, M.—(1941) in 'Dental Caries: Findings and Conclusions on its Causes and Control'. 2nd ed. New York (American Dental Association), p. 241.
MUSCHETT, C. W.—(1956) *Amer. J. clin. Nutr.*, **4**, 326.
RINEHART, J. F. AND GREENBERG, L. D.—(1956) *Ibid.*, **4**, 318.
Idem AND ABDUL-HAJ, S. K.—(1951) *Arch. Path.* (*Lab. Med.*), **52**, 189.
SCHROEDER, H. A.—(1955) *J. Chronic Diseases*, **2**, 28.
SCHULTZ, A. H.—(1935) *Amer. J. Phys. Anthrop.*, **19**, 489.
SHAW, J. H., ELVEHJEM, C. A. AND PHILLIPS, P. H.—(1945) *J. dent. Res.* **24**, 129.
STEINMAN, R. R. AND HARDINGE, M. G.—(1958) *Ibid.*, **37**, 874.
STREAN, L. P., BELL, F. T., GILFILLAN, E. W., EMERSON, G. A. AND HOWE, E. E.—(1958) *N.Y. St. dent. J.*, **24**, 133.
Idem, GILFILLAN, E. W. AND EMERSON, G. A.—(1956) *Ibid.*, **22**, 325.

SURVEY OF PAPERS

MOVAT, MORE and WOLOCHOW investigated the origin of fibrinoid material following different types of injury and have assessed the significance of the presence or absence of plasma cells in the lesions produced.

Using rabbits, they induced mechanical injury by incision, chemical injury by the injection of trypsin, and physical damage by the implantation of radon seeds into the flanks of the animals.

The mechanical injury was followed by haemorrhage and fibrin was deposited along the cut edges and in the depths of the wound. It was found that the inspissation of the plasma resulted in the formation of typical fibrinoid material but " fibrinoid degeneration of the collagen " did not occur.

Chemical injury induced a necrotizing and inflammatory reaction with a fibrinous exudate in the walls of the blood vessels and in the adjacent connective tissue. The fibrinoid material found in the lesion was derived from the exuded plasma and not from the collagen.

Physical damage also produced a fibrinous exudate with the presence of fibrinoid material in the connective tissues derived from the inspissated plasma. Degeneration of the collagen occurred but fibrinoid degeneration of the ground substance was not seen.

This fibrinoid material was formed in all 3 varieties of injury and was therefore regarded as non-specific. The plasma cell infiltration was not seen and this was regarded as additional support for the contention that plasma cells only appear in association with antigen stimulation (p. 97).

WESTWOOD, APPLEYARD, TAYLOR-ROBINSON and ZWARTOUW have studied the effect of increasing the concentration of ERK 1 cells in suspended cell cultures with a view to increasing the concentration of poliovirus in the supernatant fluid. The highest virus titres were obtained with a cell concentration of 10^7 per ml. although the virus yield per cell was greater at a lower cell density (p. 105).

DAY investigated the removal of cholesterol and cholesteryl esters from reticulo-endothelial cells. When these substances were injected intraperitoneally into rats they were absorbed *via* the diaphragmatic lymphatics and retained in the sternal lymph nodes.

He found that almost all the cholesterol taken up was removed from the lymph nodes within 10 days. The ingestion of un-esterified cholesterol by the nodes was followed by an increase in cholesteryl ester, while the ingestion of cholesteryl esters was followed by an increase in free cholesterol. The uptake of cholesterol and cholesteryl oleate also resulted in the accumulation of phospholipids and total fatty acids in the lymph nodes. The author considered that metabolic changes may influence the removal of cholesterol from the reticulo-endothelial cells (p. 112).

CORNELIA HOCH-LIGETI has studied the progressive degeneration of kidney and liver function which follows the injection of 6-dimethylamino-9 (3′ amino β D ribofuranosyl) purine into rats. Gross disturbance of protein and of lipoid metabolism has been observed as nephrosis developed (p. 119).

BAUER has demonstrated a linear relationship between the reciprocal of the survival time in mice and the logarithm of the dilution of a virus suspension with which they have been inoculated. This applies to several viruses, and the intercept of the regression line with the abscissa is D_0, the point of zero mortality. For a given host virus system this has a constant relationship to the LD50 to which it is preferable ; for fewer animals are used to determine it, and the information obtained with every animal is relevant (p. 130).

THELMA BAXTER using a micro-dissection technique studied the changes in rabbits' kidneys induced by cortisone. Lesions were present in the kidneys of the cortisone-administered rabbits but the changes were more marked in those animals put on a special " rachitic " diet. The glomeruli were enlarged with an increase in the capsular spaces and swelling of the tufts. There was dilatation of the tubules and casts were formed by red cells, pigmented and fatty material. Fat was also found in the glomeruli and the tubules. The author discusses

whether these changes are due to the direct action of cortisone on the renal tissue or to altered ion balance (p. 140).

PLATT has used grafting experiments in guinea-pigs to study the relationship between susceptibility to damage by the virus of foot-and-mouth disease and the source and site of epithelial tissue. The usual predilection sites were thought to be conditioned largely by associated hyperplasia and persistent irritation since grafting to other areas greatly reduced susceptibility whereas hairy skin transfered to the tongue or metatarsal pad became susceptible (p. 150).

MELLETT, WALTER and HOUGHTON investigated ulceration of the skin of the guinea-pig following local irradiation.

Twenty-two young adult albino guinea-pigs received 6,000 r in a single dose of 3 min. duration to each flank. Two phases of ulceration were observed; the first phase resulted in superficial ulceration from the 18th day onwards and consisted of loss of epithelium with little damage to the underlying tissues; this ulcerated area sometimes healed by hyperplasia of the epithelium but all were followed by the second phase of deep ulceration. In this phase there was necrosis of the deep tissues with vascular changes including fibrinoid necrosis and thrombosis. From this evidence they concluded that the pathogenesis of the ulceration differs in the two phases (p. 160).

BRIDGES induced gliomata in mice by the intracerebral implantation of methylcholanthrene, and then studied the effects of dietary factors on survival times.

It was found that the incidence of glioma formation was not influenced by dietary deficiency but those animals deficient in thiamine survived the intracerebral implantation by a significantly shorter time than did animals not deficient in thiamine. The author suggests that the thiamine deficient animals died sooner due to a greater tendency for haemorrhage to occur into the substance of the gliomata (p. 169).

SOURANDER, LYCKE and EBBA LUND have observed human cancer and embryo kidney cells in culture on the warm stage of the phase contrast microscope, and watched the infection of the cells by *Toxoplasma gondii* and the subsequent multiplication of the parasites. These divided 2–3 hr. after inoculation and then about once per hr. The new clones of parasites so formed lay in clear zones in the cell cytoplasm, usually near the nucleus. Cells ruptured 2 days or more after infection (p. 176).

VICARI, OLITZKI and ZAHAVAH OLITZKI have observed the effect of heat labile toxin extracted from the Bukarest R strain of *Sh. dysenteriae* on human liver, monkey kidney and KB cells in culture. They found all these cells were visibly damaged by a dose of toxin less than 0·001 of the LD50 for mice, and were sensitive indicators of traces of toxin in toxoid. Small quantities of antitoxin could be detected by neutralization tests in cultures of these cells (p. 179).

THORBECKE, MAURER and BENACERRAF have studied the phagocytosis in mice of bovine serum albumin (BSA), and of denaturation products of BSA obtained with various physical and chemical agents. Their methods were to estimate the uptake of denatured BSA, injected intravenously and labelled with ^{131}I by liver, spleen, kidney and lung; and to measure the interference caused by these substances with phagocytic clearance of carbon from the blood. Large size and changed surface charge predisposed molecules to phagocytosis (p. 190).

BERDJIS, GREENBERG, RINEHART and FITZGERALD describe the high incidence of oral and dental lesions that occurred in 29 rhesus monkeys which had been subjected to varying degrees and periods of pyridoxine deficiency.

Rhesus monkeys maintained on deficient pyridoxine diets for periods in excess of 16 months showed a high incidence of dental caries in addition to deformed, shortened and mal-aligned teeth and a failure of development of the canine teeth.

The pyridoxine deficiency appeared to exert its deleterious effects on the permanent teeth during the course of their development.

Glossitis and gingivitis were also observed in these animals. The possible relationship of these findings to human dental caries is discussed and the possibility that the cariogenic effect of pyridoxine deficiency might be associated with an alteration of the oral bacteriological flora (p. 198).